

Functional impact of microRNA-34a on stem cell differentiation towards smooth muscle cell

By Xiaotian Yu

Supervisors: Professor Wen Wang

Dr Qingzhong Xiao

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Statement of originality

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Details of collaboration and publications:

Human ES cell culture and treatment were carried out by Dr. Qingzhong Xiao and Dr. Zengling Luo. Dr. Qingzhong Xiao also helped me for experiments pertaining to flow cytometry and cell sorting. Dr. Guanmei Wen performed the *in vivo* Matrigel experiments. mRNA microarray assays were carried out with the help from Dr. Li Zhang at Zhejiang University. Dr. Yuan Huang, Mr. Qishan Chen and Mr. Hanqing Zhao helped me in creating miRNA reporters. RNA IP experiments were carried out with the help from Dr. Le Anh Luong. Lastly, Mr. David Sims and I worked together when performing collagen type I induced SMC differentiation.

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Abstract

MicroRNAs play an important role in biological regulation. Recently miR-34a has been reported to regulate tumour cell cycle progression and apoptosis. However, the functional role of miR-34a in smooth muscle cell (SMC) differentiation from stem cells is yet unclear. Main objectives of this PhD project are to determine the functional role of miR-34a and its target genes in SMC differentiation and underlying mechanisms.

Mouse embryonic stem (ES) cells were seeded on collagen coated flasks in differentiation medium to allow SMC differentiation. Upon analysis, miR-34a was significantly up-regulated during SMC differentiation. Results demonstrated that overexpression of miR-34a significantly promoted SMC-specific gene expression, while knockdown of miR-34a inhibited expression of SMC specific gene. Enforced expression and knockdown of miR-34a in differentiating ES cells up-regulated and down-regulated, respectively, several SMC transcription factors in a similar manner. It was also found that miR-34a overexpression in stem cells promoted SMC differentiation *in vivo*. Furthermore, deacetylase sirtuin 1 (Sirt1) was identified as one of the top targets of miR-34a. Surprisingly, Sirt1 was demonstrated to be positively regulated by miR-34a during SMC differentiation in a cellular context and RNA sequence dependent manner.

Mechanistically, the data suggested that miR-34a promoted differentiating stem cells arrest at G0/G1 phase, and a significant decreased incorporation of miR-34a and SirT1 RNA into Ago2-RISC complex was observed upon SMC differentiation. The results demonstrated that Sirt1 acted as a transcriptional activator in the regulation of SMC gene during ES cell differentiation. Finally, H3K9 tri-methylation around the promoter regions of the SM α A and SM22 α genes was also found to be significantly inhibited by SirT1 overexpression.

These findings suggest that miR-34a plays an important role in SMC differentiation from ES cells. Meanwhile, Sirt1 can be regulated by miR-34a through an unexpected pathway and it was identified as a functional modulating target in miR-34a mediated SMC differentiation.

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List of Abbreviation and Acronyms

2D	2-dimensional
3D	3-dimensional
AGO	Argonaute
ARE	AU-rich
atRA	<i>all-trans</i> RA
BM	Basic medium
BMP	Bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
Ccnb	Cyclin B1
Cdx4	Caudal type homeobox 2
Chip	Chromatin immunoprecipitation
CM	ES cell culture medium
DAPI	4',6-diamidino-2-phenylindole
DGCR8	DiGeorge syndrome critical region gene 8
DM	SMC differentiation medium
DMSO	Dimethyl sulfoxide
dsRNA	Double-stranded RNA
EB	Embryoid-body
ECM	Extracellular matrix

ES cell	Embryonic stem cell
FBS	Fetal bovine serum
FXR1	Fragile X mental retardation–related protein 1
GFP	Green fluorescent protein
ICM	Inner cell mass
IL	Interleukin
iPSC	Induced pluripotent stem cell
Klf4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor
MECP2	Methyl CpG binding protein 2
MEF2C	Myocyte-specific enhancer factor 2C
MEFs	Mouse embryonic fibroblasts
miRNA	Micro RNA
Nox4	NADPH oxidase 4
OCT	Octamer-binding transcription factor
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR β	Platelet-derived growth factor receptor beta polypeptide
PGC	Primordial germ cells
Pri-miRNA	Primary miRNA

RA	Retinoic acid
RISC	RNA-induced silencing complex
RNA-IP	RNA immunoprecipitation
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
Sca-1+	Stem cell antigen-1 positive
SEM	Standard error of the mean
Sirt1	Deacetylase sirtuin 1
SM22 α	Smooth muscle 22 α
SMC	Smooth muscle cell
SM-MHC	Smooth muscle myosin heavy chain
SM α A	Smooth Muscle α -Actin
SOX2	(Sex determining region Y)-box 2
SRF	Serum response factor
SSC	Somatic stem cell
SSEA	Stage-specific embryonic antigen
STAT3	Signal transduction and activation of transcription 3
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRBP	Tar RNA binding protein

UTR	Untranslated region
VEGFR	Vascular Endothelial Growth Factor Receptor
VSMCs	Vascular smooth muscle cells
XPO5	Exportin-5
ZEB1	Zinc finger E-box-binding homeobox 1

CHAPTER 1 Introduction and background

1.1 Introduction

Regenerative medicine and tissue engineering are an rapid developing field with the ultimately goal to repair, replace, or regenerate cells, tissues, or organs that are lost or damaged due to disease, injury, or ageing. Recent advances in this field have been greatly influenced by better understanding in multiple areas, including stem cell biology, tissue turnover and replacement in adult mammals, and embryonic development. Pluripotent stem cells including embryonic stem (ES) cells have been under intensive investigation for using in regenerative medicine because they hold significant potential for clinical therapies due to their distinctive capacity to both unlimited self-renew and differentiate into a wide range of specialized cell types, including vascular endothelial cells (Zeng et al., 2006, Xiao et al., 2006) and smooth muscle cells (SMCs)(Xiao et al., 2007, Xiao et al., 2012, Pepe et al., 2010, Huang et al., 2013). SMCs are major component of blood vessel wall and responsible for structure and contract ability of healthy blood vessel. Furthermore, SMC differentiation is a critical process during cardiovascular system development, and such differentiation is also involved in SMC proliferative related cardiovascular diseases, such as atherosclerosis and hypertension. Therefore, understanding the

transcriptional regulatory circuitry of SMC differentiation is fundamental to understanding human cardiovascular system development and realizing the therapeutic potential of these cells. However, the detailed molecular mechanisms of SMC differentiation from stem cells have not been fully clarified.

MicroRNAs (miRNAs) are highly conserved, endogenous, short non-coding RNAs (usually contains 22 nucleotides). miRNAs have been recently identified in almost all species and recognized as important biological regulators in tissue development, cellular homeostasis, and diseases. The “classical” thinking of miRNAs’ function is that mature miRNAs work together with RNA-induced silencing complex (RISC), to suppress their target genes expression by inducing mRNA cleavage, mRNA decay or by inhibiting mRNA translation (Bagga et al., 2005, Giraldez et al., 2006, Filipowicz et al., 2008). However, recent studies suggested that instead of repression, miRNAs can up-regulate their target genes expression under specific circumstance (Vasudevan et al., 2007, Vasudevan et al., 2008). Importantly, Landgraf et al. (2007) reported that miRNAs are expressed in a tissue specific manner, indicating that miRNAs may play an important role in cell/tissue specification. Studies also suggest that miRNAs are involved in ES cells regulation, cardiovascular development and SMCs differentiation and proliferation. Dicer and Drosha are two rate-limiting enzymes involved in miRNA biogenesis. Murchison et al. (2005) and Wang et al (2007)

demonstrated that miRNAs play critical roles in stem cell self-renewal and differentiation by disrupting Dicer or Drosha expression. Loss of miRNAs in Dicer-deficient mice caused severe impairment of heart and blood vessel development, indicating the significance of miRNAs in cardiovascular development (Yang et al., 2005). In addition, Albinsson et al. (2010) further highlighted the importance of miRNAs in SMC growth, differentiation and function by pointing out that deletion of Dicer in vascular smooth muscle led to decreased SMC proliferation and differentiation, and caused thinner vessel walls, impaired contractility and hemorrhage due to the global loss of miRNAs in vascular smooth muscle. However the exact role of individual miRNAs in SMC differentiation from ES cell remains to be further investigated.

This project demonstrates for the first time that miR-34a play an important role in mouse and human ES cell differentiation towards SMC lineage *in vitro* and *in vivo*. More important, data from this project suggests that instead of repression, miR-34a positively regulates its target gene deacetylase sirtuin 1 (SirT1), which has been identified as a potential transcriptional activator of SMC genes in this project.

1.2 Stem cell

Stem cells have the ability to unlimited self-renew and undergo differentiation into various cell lineages (also known as pluripotent). Pluripotent stem cells have been under intensive study in the past years, because they hold great promise to be used as the critical tool in regenerative medicine and tissue engineering, for clinical cell therapy to treat current devastating and incurable diseases, such as type 1 diabetes, blindness, damaged articular cartilage, neurological diseases and terminal cardiovascular diseases. Also, stem cells provide a tractable, manipulable and cost-efficiency *in vitro* cell culture experimental system for researchers to study tissue development, genetics, pathology and physiology, due to their nearly unlimited sources (self-renew) and pluripotency, as compared with more expensive, hard to obtained *in vivo* materials.

1.2.1 Categorization of stem cells

Currently, stem cells are categorized in to three groups based on their sources: embryonic stem cells, adult stem cells and induced pluripotent stem cells (iPSCs) (Evans and Kaufman, 1981, Takahashi and Yamanaka, 2006). Adult stem cells, also known as somatic stem cells (SSCs), are “partially” pluripotent stem cells that found in tissues and organs, including skin, brain, bone marrow, intestine and neural tissue.

SSCs are primarily involved in maintaining and repairing/regenerating specific tissue, and under certain circumstances they may be involved in diseases development (Phinney and Prockop, 2007). SSCs used for potential stem cell therapy are autologous, as they are usually harvested from the patients hence there is almost no immunologic barriers. However, unlike ES cells, the pluripotency of SSCs is limited due to the fact that they are lineage commitment, which means they only hold the capacity to differentiate into specific cell lineages from given germ layer based on their origins. Furthermore, SSCs are limited in quantity due to their lower capacity to divide during *in vitro* culture. Evidently, some specific SSCs are hard to obtain from patients (Barrilleaux et al., 2006, Pittenger et al., 1999).

In 2006, Takahashi and Yamanaka (2006) reported that by transducing four key transcription factors: OCT4, SOX2, c-Myc and KLF4 into mouse somatic cells such as adult fibroblasts, these cells can exhibit the morphology and growth properties of ES cells, as well as expressing ES cell marker genes, designated as iPSCs. iPSCs were then successfully generated from human somatic cells by transduction of same transcription factors since 2007 (Takahashi et al., 2007, Park et al., 2008). Further analysis demonstrated that iPSCs not only expressed pluripotent markers, but also have the similar function of ES cells and hold the ability to differentiate into cell types from three germ layers, and contribute to embryonic development (Maherali et

al., 2007, Takahashi et al., 2007, Takahashi and Yamanaka, 2006). Since then, several studies have reported refined process of reprogramming iPSCs, including adjust transcription factors and add extra enhancers (Park et al., 2008, Huangfu et al., 2008, Judson et al., 2009).

The recent achievements in charactering the full potentials of various iPSCs generated by using different techniques have advanced one step closer to patient-specific cell therapy of diseases, and construct *in vitro* models of specific human tissue development and diseases, especially for the cases where animal models cannot fully represent human models (Puri and Nagy, 2012). Patient-specific iPSCs generation means there is highly reduced immunologic barriers and ethical issues. Also, compared with SSCs, the materials for generating iPSCs are easier to obtain from patients. However, despite the advantages of iPSCs and the rapid development of iPSCs research area, there are still some concerns regarding the function or application of iPSCs. Although iPSCs do share similar pluripotent and other properties as ES cells, the understanding of iPSCs is still far from complete, including the mechanism of reprogramming and characterization of iPSCs (Puri and Nagy, 2012). Furthermore, the success rate of generating iPSCs was still low (Takahashi and Yamanaka, 2006), and iPSCs displayed a lower efficiency in neuroepithelia differentiation, as compared with ES cells (Hu et al., 2010). Also,

another major concern of iPSCs in clinical therapy is their tumorigenicity, as suggested by some studies (Knoepfler, 2009).

1.2.2 Embryonic stem cells

ES cells are pluripotent stem cells derived from inner cell mass of blastocyst, an early stage embryos approximately 3.5 days post coitum (Figure 1). Mouse ES cells were first isolated in 1981, and their pluripotency was demonstrated by their contributions to all the tissue developments in mice after injecting isolated ES cells back into host blastocysts (Evans and Kaufman, 1981, Martin, 1981, Bradley et al., 1984). After seventeen years, human ES cells were isolated in 1998 (Thomson et al., 1998). The isolation of ES cells have dramatically benefit researches in tissue development and regenerative medicine in the past years, due to the facts that ES cells have almost an unlimited self-renew potential and are capable of differentiating into all cell types from all the three germ layers (Figure 1).

As self-renew and pluripotency are the important features of ES cells, the first challenge in ES cells based research is to understand the mechanism by which controls self-renew and pluripotency of ES cells. Mouse ES cells were initially cultured and maintained with mouse embryonic fibroblasts (MEFs), which indicated that MEFs may provide the essential factors to maintain the undifferentiated ES cells.

Later several studies revealed that MEFs feeder-cell-derived leukemia inhibitory factor (LIF) is the critical factor in maintaining ES undifferentiated ES cells (Williams et al., 1988, Smith et al., 1988, Stewart et al., 1992). A more recent study by Ying et al. (2003) pointed out that bone morphogenetic protein (BMP4) can replace serum in the presence of LIF, to support ES cell culture. LIF is a soluble glycoprotein of interleukin (IL)-6 family of cytokines, and functions through regulate signal transduction and activation of transcription 3 (STAT3) with gp130 signaling complex (Niwa et al., 1998, Matsuda et al., 1999, Wobus and Boheler, 2005). BMP4 support undifferentiated ES cells via Smad activation and inductions of helix-loop-helix Id factors (Keller, 2005). More recent studies showed that three transcription factors: Nanog, OCT3/4 and SOX2 are key factors that control self-renew and pluripotency of ES cells (Mitsui et al., 2003, Chambers et al., 2003, Niwa et al., 2000). OCT3/4 and SOX2 can form a complex that suppresses expression of Cdx2, and OCT4 is reported to interact with BMP4 pathway (Niwa, 2007, Wang et al., 2012, Niwa et al., 2005). Nanog is suggested to inhibit primitive endoderm and mesoderm differentiation through regulating Gata6 and brachyury, respectively, and study showed that Nanog is downstream effector of LIF-STAT3 pathway (Mitsui et al., 2003, Suzuki et al., 2006, Pan and Thomson, 2007, Nishikawa et al., 2007). More important, OCT3/4, Nanog and SOX2 can bind to each other's promoter so that they form a core network to regulate ES cells, which involving various signal pathways

(Pan and Thomson, 2007).

OCT3/4, SOX2 and Nanog play important roles in regulating self-renew and pluripotency of ES cells, and they are usually referred as stem cell undifferentiated markers. Regulation of undifferentiated ES cells is controlled by a complex network, and studies also revealed more ES cells regulator/markers, such as KLF-4, SSEA-1, SSEA-3/4 and GCTM-2 (Solter and Knowles, 1978, Henderson et al., 2002, Zhang et al., 2010, Reubinoff et al., 2000). Although OCT3/4, SOX2 and Nanog are expressed in both mouse and human ES cells, their functions and signal pathways may be slightly different in these of ES cells. Furthermore, some of the stem cell makers, such as SSEA-1, only expressed in mouse ES cells, while some others (e.g. SSEA-3/4) are detected in human ES cells (Wobus and Boheler, 2005).

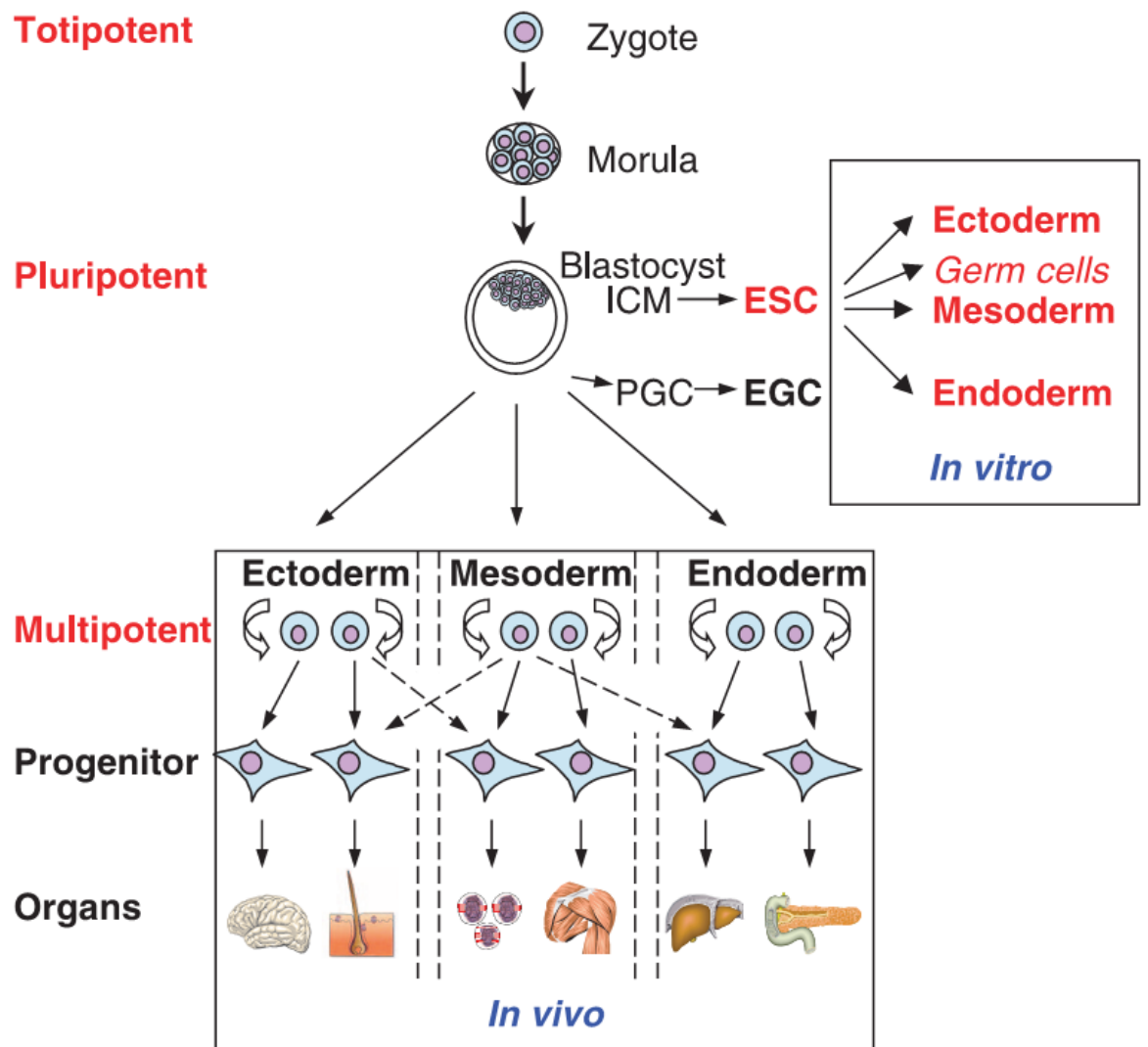


Figure 1 Stem cell hierarchy depicting the development from Zygote to Organs.

ES cells are isolated from ICM at blastocyst stage. ICM develops into three primary germ layers: ectoderm, mesoderm and endoderm, as well as primordial germ cells (PGC). Specific progenitor cells are derived from relative germ layers, and further differentiate into various types of somatic cell, which form the organs(Wobus and Boheler, 2005).

1.2.3 ES cell culture

Two methods are commonly used for *in vitro* ES cell culture: ES cells are either cultured with feeder cells (in most of the case, MEFs are used as the feeder), or cultured on protein matrices with defined culture medium combined with LIF, Fetal bovine serum (FBS) and/or BMP4. ES cells cultured with inactive MEFs are the “traditional” way of maintaining undifferentiated ES cells. As mentioned earlier, MEFs can secrete factors that maintain undifferentiated ES cells; they also provide mechanical support for ES cell growing. ES cell cultured with MEFs is a well refined method and the outcome has been well validated in the past years (Williams et al., 1988, Stewart et al., 1992). However, it requires additional routinely maintaining of MEFs in the lab, and raise the risk of MEFs contamination. Another method is culturing ES cells on protein matrices (gelatin is well commonly used as the base layer), and use defined medium in supplemented with recombinant LIF and FBS, without feeder layer. Maintaining mouse ES cells in feeder free condition has achieved great success over the past years with the presence of LIF. On the other hand, LIF is not sufficient for maintain human ES cells (Daheron et al., 2004, Humphrey et al., 2004). Human ES cells feeder free culture was reported by using MEF conditioned medium in 2001, although it is more complex than culturing mouse ES cells and usually requires basic Fibroblast Growth Factor (Xu et al., 2001, Amit et al., 2000). As compared with culturing on MEFs, the feeder-free culture method

requires less work to maintain ES cells and reduces the risk of contamination. However, it has some drawbacks. The culture medium must be well defined to ensure that it provide essential components to inhibit ES cell differentiation and support cell growing. This method usually requires presence of FBS to provide nutrients for ES cells; however the constituents of FBS are not fully defined, even after screening, which potentially induces differentiation. Even though mouse ES cell feeder free culture is well established, human ES cell culture in feeder free condition may not be as efficiency as culturing with MEFs and the cost of human ES cell feeder free culture is relatively high due to the requirements on the medium and supplements.

1.2.4 ES cell differentiation *in vitro*

ES cells are regulated by a complex, “balanced” network to maintain their pluripotency through various signal pathways, and the mechanism has been under intensive study since ES cells were discovered. Compared with the understanding on maintaining pluripotency, ES cell differentiation, especially the mechanism and process, is still poorly defined. Withdraw of factors that maintain their stem cell function in cell culture, for example removal of LIF in mouse ES cell culture, usually triggers ES cell differentiation. However, ES cell differentiation is induced by a variety of stimulations, such as growth factors, extracellular matrix, mechanical stress and chemical stimulations (Niwa, 2007, Nishikawa et al., 2007).

Understanding ES cell differentiation *in vitro* and *in vivo* has always been a major challenge in stem cell research.

Currently, there are at least three common used approaches to induce ES cell differentiation *in vitro*: embryoid-body (EB) culture, feeder-cell culture and extracellular matrix proteins culture, and each method has its own advantages and drawbacks (Figure 2).

EB culture

The concept of EB culture is to mimics *in vivo* germ layer formation from ICM and it was first used in 1975 for teratocarcinoma cells differentiation (Martin and Evans, 1975). EB culture was then used on ES cell differentiation from 1985 by several studies (Doetschman et al., 1985, Keller, 1995). In EB culture, supplements that maintaining pluripotency of ES cells were removed, and ES cells were allowed to aggregate and develop into three dimensional colonies known as EBs. EBs contains visceral endoderm in the outlayer with primitive ectoderm derived lineages inside, although the structures of EBs are far less organized than actual *in vivo* embryo. Most of the cell lineages including neuronal and mesoderm lineages, as well as germ cell precursors can be induced by EB culture, as reviewed by Nishikawa et al. (2007). EB culture is the most similar representation of *in vivo* ES cell differentiation model,

and providing one of the best systems for some researches, i.e. in tissue development area. The initial induction of ES cell differentiation by EB culture is relatively easy, however it is extremely difficult to conduct guided differentiation in EB culture, and monitoring of cell behavior is tricky due to their 3D structure. Also, EB culture is quite time-consuming, as compared with other two methods.

Feeder-cell culture

Similar to maintaining ES cells on MEFs, ES cells are cultured on selected stromal cells. Differentiation of ES cells is supported by the surrounding feeder cells and given cell culture medium. OP9 stromal cell line is one of the most commonly used cell line, which can induce mesoderm lineages differentiation from ES cells (Nakano et al., 1994). The guided/selective differentiation in this method is slightly easier compared with EB culture. PA6 stromal cell line was reported to induce selective neuronal lineage differentiation from ES cells (Kawasaki et al., 2000). The drawbacks of this method are that the undefined factors generated by the feeder cells may affected the ES cell differentiation, and it requires additional maintenance of stromal cells. Furthermore, sometimes it is difficult to isolate differentiated ES cells from stromal cells. Even through guided differentiation is theoretically possible in this method; it still remains as a challenge.

Extracellular matrix proteins culture

In this method, ES cells are cultured in a monolayer of selected extracellular matrix proteins (also known as substrates) with highly refined cell culture medium and supplements (Nishikawa et al., 1998). In order to conduct guided ES cell differentiation, the culture medium usually contains defined supplements which potentially induce specific cell lineages differentiation, and the selection of substrates is critical for guided ES cell differentiation. This method minimum the influence of neighboring differentiating cells from EB culture, as well as that from stromal cells. The highly defined cell culture conditions are critical for investigating signal pathways. Furthermore, it is up to now the best method to induce selective ES cell differentiation and obtain high purity of specific differentiated cell types. Once the differentiation model is established, it is relatively easy to use such a model study the molecular mechanisms of cell differentiation with high reproducibility. It has been reported that ES cells can differentiate into smooth muscle cells and endothelial cells by using this method, respectively (Xiao et al., 2006, Luo et al., 2013). However, it has its own drawbacks. Establishing the specific differentiation model is a huge challenge, which requires continuing studies in this field and multiple repeated trials to ensure its efficiency. As a result, the numbers of specific ES cell differentiation induced by this method are still limited. Although the cell culture conditions are the most defined among these three methods, it still cannot fully eliminate the undefined

factors involved in the differentiation, such as that from FBS or subsequent changes of the cells. The requirements for culture medium is very high, as well as the supplements, which may significantly increase the cost.

With the recent development of engineering and material fields, more new techniques are used in maintaining undifferentiated stem cell, or inducing differentiation. For example, with the fast growing nano-technology, it has been reported that surface nanotopography has significant impact on stem cell self-renew and differentiation (Bae et al., 2014, Chen et al., 2012). Combining these new techniques with currently stem cell culture system will surely push forward the understanding in this field.

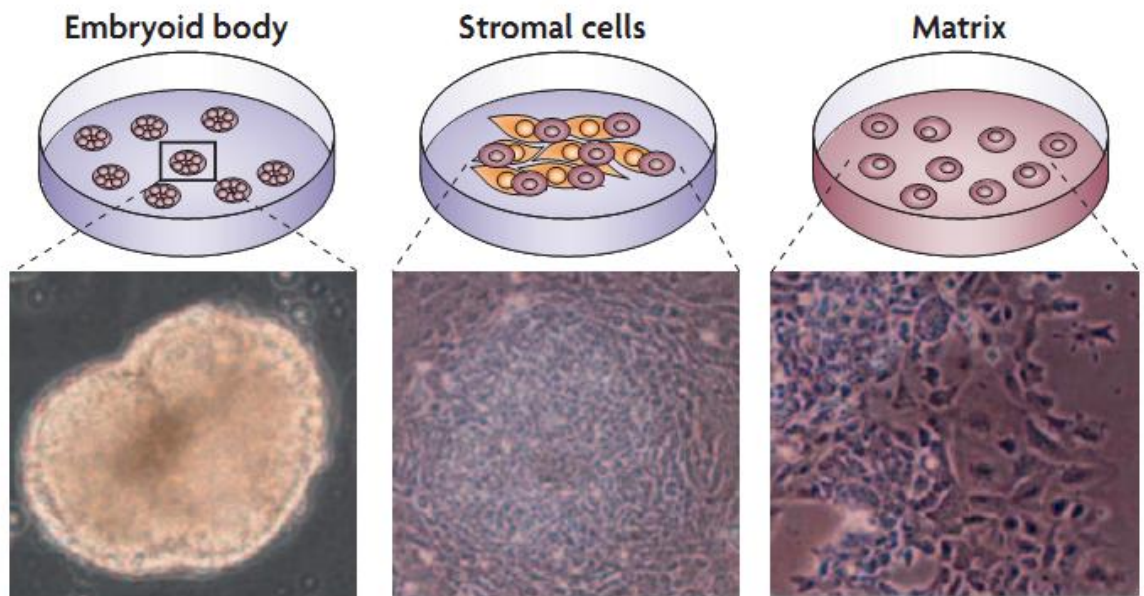


Figure 2 Three commonly used approaches for ES cell differentiation *in vitro*.

There are three commonly used methods to induce ES cell differentiation *in vitro*. In EB culture (left) ES cells are allowed to aggregate into embryo-like EBs. Method using Feeder-cell (middle) cultured ES cells on selected stromal cells. While in extracellular matrix culture (right) ES cells are cultured on substrates under highly defined conditions (Nishikawa et al., 2007).

1.3 SMCs and their differentiation from stem cells

1.3.1 Smooth muscle cell

Smooth muscle is an involuntary non-striated muscle and it can be found from various locations of human body, such as blood vessel walls, lymphatic vessels, bladder, uterus and skins. Smooth muscle cells located in blood vessels, such as in large and small arteries and veins, are usually termed as vascular smooth muscle cells (VSMCs).

Initially derived from mesoderm, mature VSMCs originate from various sources of progenitors. VSMCs provide structural integrity and contribute to contraction and relaxation of the blood vessels, which alter blood vessel diameter. They also contribute to adjust blood flow and blood pressure. Unlike other muscle cells, VSMCs are not terminally differentiated in human tissues (Velican and Velican,

1980). VSMCs can switch between contractile and synthetic phenotypes, with different morphology, proliferative ability, migration properties and expression levels of SMC gene markers.

Besides providing structural and functional support to blood vessels, VSMCs proliferation, migration and differentiation from stem/progenitor cells are found to play an important role during vessel remodeling, post vascular injury and diseases, such as atherosclerosis (Owens et al., 2004, Ross, 1999, Xu, 2006). With the advanced in tissue engineering, VSMCs hold the potential ability to reconstruct human vascular tissue for clinical treatment. However, the limited lifespan of VSMCs may become the barrier for the cell therapy applications. Thus, better understanding of SMC differentiation from stem cell will benefit the recognition of diseases, tissue development and clinical tissue engineering applications.

1.3.2 SMC differentiation from stem cell *in vitro*

Several *in vitro* SMC differentiation model have been established to study the process of SMC differentiation from stem cells, and/or obtain functional SMCs (Xie et al., 2011, Xiao et al., 2010). P19 cell line was reported to have similar mechanism and features to ES cells for differentiation (Rideg et al., 1994). It has been reported that expose P19 cells to retinoic acid (RA) for 48 hours or treat with dimethyl

sulfoxide (DMSO) can induce these cell differentiate into fibroblast-like cells. The differentiated cells express SMC marker SM α A, and study also showed that they respond to angiotensin II and endothelin (Drab et al., 1997, Sinha et al., 2004). A404 cells, which are derived clonal cell line from P19 but have been genetically modified by harboring a SM α A promoter/intron-driven puromycin resistance gene (Manabe and Owens, 2001). Exposing these cells to *all-trans* RA (atRA) can induce SMC differentiation. More importantly, the differentiated SMC-like cells can be sorted out due to the presence of puromycin resistance gene, and results suggested that more 90% of cells express SMC markers, such as SM α A, calponin or smooth muscle myosin heavy chain (SM-MHC)(Manabe and Owens, 2001, Spin et al., 2004). Although P19 and A404 cell lines have similar differentiation ability to ES cells, and they have been successfully demonstrated to differentiate into SMC-like cells. However they are not “pure” ES cells. A404 cell lines are genetic modified with SM α A promoter and purmycin resistance genes, thus using these models is inappropriate for studying the mechanism of SMC differentiation from original ES cells. Furthermore the differentiation ratio from P19 cell line was reported very low (Manabe and Owens, 2001, Xie et al., 2011).

Mouse ES cells have been reported to differentiate into SMCs by using EB culture. After EB formation they were treated with atRA and dibutyryl-cAMP, and results

suggested that 67% of the cells in the EBs express SMC-like features, compared with 10% in the control group (Drab et al., 1997, Sinha et al., 2004). EB culture can also be used in SMC differentiation from human ES cells. CD34⁺ vascular progenitor cells isolated from human ES cells can differentiate into SMC-like cell with treatment of platelet-derived growth factor-BB (PDGF-BB). These SMC-like cells express SM α A, SM-MHC, SM22 α and calponin. However, they also express some endothelial markers (Ferreira et al., 2007, Xie et al., 2007, Xie et al., 2011).

Recent studies also reported that SMC differentiation from stem cell can also be achieved by using substrates culture combined with defined culture medium and supplements. ES cells cultured on a monolayer and treated with atRA were induced to SMC differentiation, and up to 65% of cells expressed SM-MHC. They also express other SMC makers, as well as some SMC functions, such as contraction response to muscarinic agonist carbachol (Huang et al., 2006, Xie et al., 2009). ECM protein collagen type IV is also reported to induce SMC differentiation from stem cells. VEGFR2⁺ progenitor cells derived from human ES cells cultured on collagen type IV in the presence of PDGF can lead to SMC differentiation (Yamashita et al., 2000, Sone et al., 2003). Xiao et al. (2007) reported that ES cells can differentiate into SMCs by using ECM protein collagen type IV. ES cells were cultured on collagen type IV coated cell culture flaks or dishes and over 50% of the cells were

SM α A positive cells after 6 days. Furthermore, stem cell antigen-1 positive (Sca-1+) cells were isolated from ES cells cultured on collagen type IV coated flasks for 3 to 4 days, and cultured with or without PDGF-BB for 6 to 90 days. Data revealed that a highly purified SMCs (>95%) expressed high SMC markers, but not endothelial cell-specific markers, after 30 days of treatment.

In summary, different methods for SMC differentiation from stem cells, with the aim to study the mechanism and/or obtaining highly purified SMCs for potential cell therapy have been reported in several studies, although each model has its own advantages and drawbacks. RA/atRA and collagen type IV are reported to induce SMC differentiation in many studies, as combined with supplements, such as PDGF or dibutyryl-cAMP.

1.3.3 Mechanisms of SMC differentiation from stem cell

SMC differentiation from stem cell is a complex process, which is governed by a interacting network, including EMC proteins and intergrins, suerm response factors (SRF) and myocardin complex, reactive oxygen species, histone deacetylases, retinoid receptors , PDGF, and TGF family and others (Xiao et al., 2010). Furthermore, SMCs is derived from various sources *in vivo*, and they can undergo phenotype switching under certain conditions, which make it very difficult to define

“terminated differentiation” in SMCs. Regardless of progress made in understanding SMC differentiation in this field, the underlying mechanism is still poorly defined.

As suggested by several studies, collagen plays an important role in SMC differentiation from stem cell. ECM is a complex tissue surrounding the cells and providing structural support as well as other important functions, such as differentiation (Lozito et al., 2009). Collagen is one of the most important components of ECM, which containing more than 16 different members. Among them, collagen I, II and III can form fibrils similar structure and collagen IV is widely found in basal lamina (Rozario and DeSimone, 2010). Collagen induced SMC differentiation from stem cell is suggested to go through collagen type IV-integrin and PDGFR β mediated signaling pathways (Xiao et al., 2007). In their study, high levels of integrin α 1, α v, β 1 and β 3 were expressed in SMCs derived from ES cells, as well as Sca-1+ progenitor cells. Inhibition of integrin function and blocking antibodies against integrin α 1, α v and β 1 significantly inhibited SMC differentiation from stem cells. Interestingly, PDGF-BB is a well-known SMC differentiation inducer (Sone et al., 2003, Yamashita et al., 2000), but the effect of exogenous PDGF-BB is weak on SMC differentiation induced by collagen IV. However, inhibition of PDGF receptor significantly inhibits SMC differentiation. The downstream signal pathways of integrin which were activated by collagen IV in

SMC differentiation include focal adhesion kinase, paxillin, phosphatidylinositol 3-kinase (PI3-kinase) and PDGFR β (Xiao et al., 2007).

TGF- β plays a critical role in activating SMC differentiation (Hirschi et al., 1998). TGF- β , PDGF-BB and ECM proteins and their secretions can activate NADPH oxidase 4 (Nox4), which further triggers a variety of downstream signal pathways (Xiao et al., 2009, Xie et al., 2011). Activated Nox4 generate reactive oxygen species (ROS), which further generate H_2O_2 and O_2^- (Deliri and McNamara, 2007, Sumimoto et al., 2005). H_2O_2 up-regulates SRF expression as well as phosphorylation. SRF can bind to CArG region of SMC gene promoter(s), where it recruits its co-activator myocardin to regulate SMC gene expression (Wang et al., 2003, Wang et al., 2004). On the other hand, O_2^- increased SRF-mediated SMC gene transcription activation through MAPK pathway in SMC differentiation (Xiao et al., 2009)(Figure 3). More recently, studies suggest that nuclear factor erythroid 2-related factor (Nrf3) can up-regulate Nox4 to generate more ROS to enhance SMC differentiation (Pepe et al., 2010, Xiao et al., 2012).

Histone modification is another important player in SMC differentiation (Tang et al., 2012, Lunyak and Rosenfeld, 2008), and methylation and acetylation are the two main forms of histone modification. Histone acetyl transferases (HATs) and histone

deacetylases determine the acetylation state of histone. Histone modification was first connected with SMC phenotype switching (McDonald et al., 2006, Xie et al., 2011). However, recently studies reported that spliced HDAC7 increase the binding between SRF and CArG region of SMC genes, by which it up-regulates SMC differentiation genes (Margariti et al., 2009).

Current studies have discovered, not all, but party of the factors that controlling the complex SMC differentiation. However, the importance of miRNAs in biological regulation has been rapidly recognized in the past years, and their functional role in SMC differentiation, is still largely unknown.

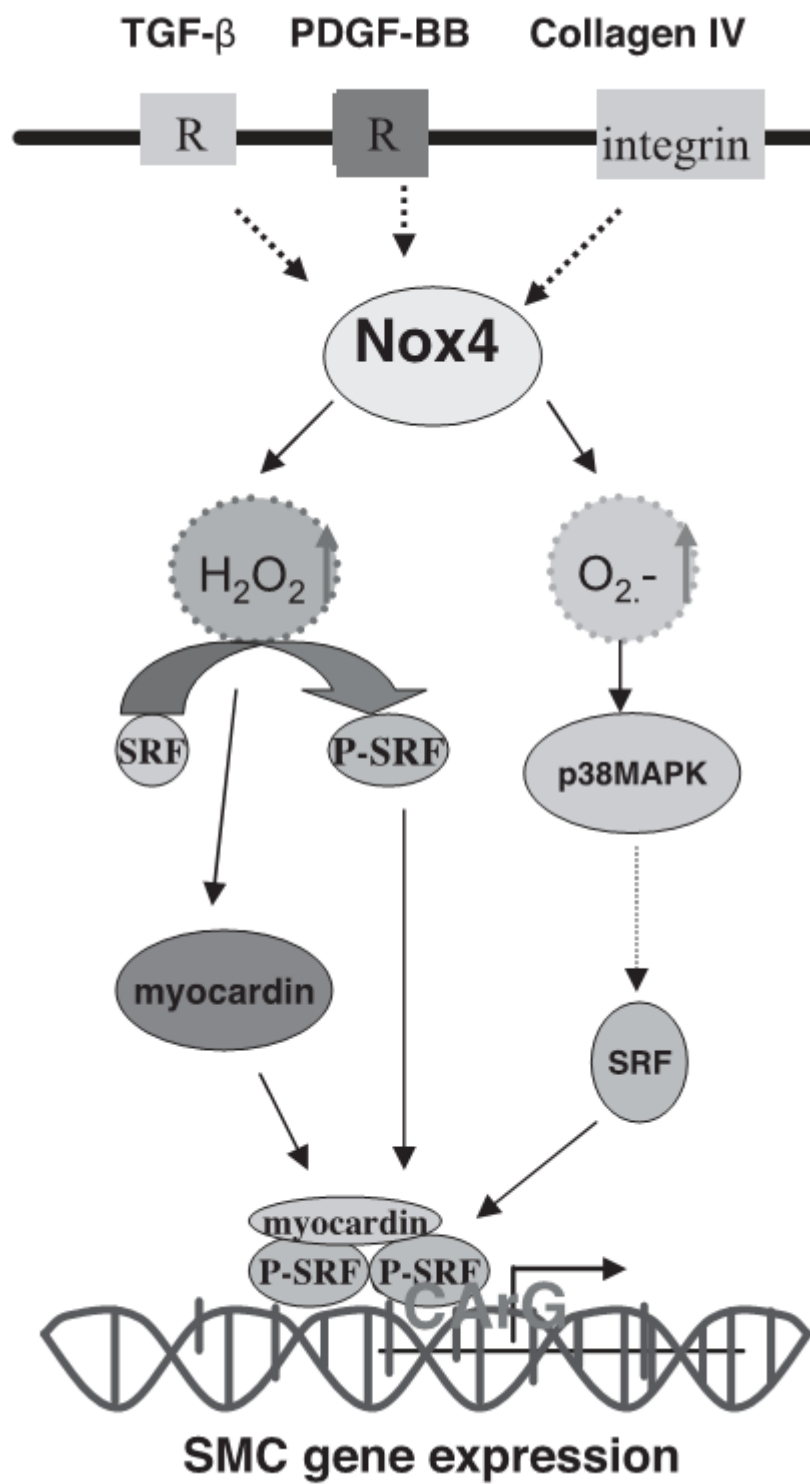


Figure 3 Role of Nox4 and signal pathways in SMC differentiation

Activated Nox4 by TGF-β or other factors generates ROS. H₂O₂ up-regulates SRF

and enhance SRF phosphorylation. Phosphorylated SRF binds to promoter region of SMC genes and work together with its co-activator myocardin to regulate SMC differentiation. O_2^- triggers p38MAPK pathways to increase SRF-mediated gene transcription activation, further enhance SMC differentiation(Xiao et al., 2010).

1.4 MicroRNA

miRNAs were first found in *C. elegans* by Lee et al(1993), and then rapidly identified in almost all species. According to version 21 of miRNA database (<http://www.mirbase.org/>), 2588 human miRNAs have been reported to the database, while 1915 mature miRNAs were found in *Mus musculus*. In the past years miRNAs have been reported to play an important role in various biological processes and diseases, such as cardiogenesis, cancer or atherosclerosis.

1.4.1 miRNA biogenesis

miRNAs are endogenous, highly conserved short non-coding RNAs (usually contains 22 nucleotides). Initially, most of the miRNAs are transcribed by RNA polymerases II (RNA Pol II) from independent miRNA genes, polycistronic transcripts that often encode multiple related miRNAs, or from introns of protein coding genes. miRNA transcription is controlled by RNA Pol II related transcription

factors and epigenetic regulators (Cai et al., 2004, Lee et al., 2004). However, it has been reported that some miRNAs are transcribed by RNA Pol III (Pfeffer et al., 2005). The initial transcription generates a long (usually over 1kb) primary miRNA (pri-miRNA), which has a stem-loop structure containing mature miRNA sequences. Within the nucleus, the pri-miRNA is processed by ribonuclease III (RNase III) type endonuclease Drosha formed complex. The ~160kDa nuclear protein, Drosha, acts specifically on double-stranded RNA (dsRNA). Drosha can directly cleave the 5' and 3' arms of the pri-miRNA hairpin, and it works together with its essential co-factor DiGeorge syndrome critical region gene 8 (DGCR8)/Pasha for processing the pri-miRNA (Han et al., 2004, Han et al., 2006). DGCR8 contains 2 dsRNA-binding domains (dsRBDs) which recognize pri-miRNA, while its C terminus interacts with Drosha. Drosha and DGCR8 form a complex called Microprocessor, which cleavages the stem-loop of the pri-miRNA to generate a small hairpin-shaped RNA with approximately 65 nucleotides in length, called pre-miRNA (Lee et al., 2003, Yeom et al., 2006, Han et al., 2006, Han et al., 2004). The majority of pre-miRNAs are generated by Drosha-DGCR8 pathway, however, it has been reported that a small amount of short intronic miRNAs (mirtrons) can bypass Drosha-DGCR8 pathway and processed into pre-miRNAs through splicing and lariat debranching enzyme (Okamura et al., 2007, Berezikov et al., 2007, Ruby et al., 2007).

Following nuclear processing, pre-miRNA is loaded into a transport complex which consists of Exportin-5 (XPO5) and GTP –binding nuclear protein RAN-GTP, and exported into the cytoplasm. GTP is hydrolysed through the nuclear pore complex and as the result, pre-miRNA is released into the cytosol from the disassembled complex (Yi et al., 2003, Ha and Kim, 2014).

In the cytoplasm, the loop of the pre-miRNA is cleaved by Dicer and generates a small (about 20-22 nucleotides) miRNA duplex (Bernstein et al., 2001, Hutvagner et al., 2001). Dicer is an RNase III endonuclease of roughly 200kDa and works specifically on dsRNAs. In human, Dicer interacts with the double-stranded RNA-binding domain proteins called Tar RNA binding protein (TRBP), to form a complex for pre-miRNA cleavage. Although TRBP is not essential for the cleavage of the pre-miRNAs mediated by Dicer, it stabilizes and enhances the process (Chendrimada et al., 2005, Haase et al., 2005, Lee et al., 2006). Also, for pre-miRNAs containing a high degree of complementarity along the hairpin stem, an Argonaute 2 (Ago2) induced endonucleolytic cleavage step takes place before Dicer mediated cleavage (Diederichs and Haber, 2007, Winter et al., 2009).

Once the pre-miRNA is cleaved by Dicer into small RNA duplex, it is loaded with an AGO protein to form the cytoplasmic effector complex called RNA – induced

silencing complex (RISC) (Hammond et al., 2001). Multiple AGO protein homologues were expressed in mammals, named AOG1-AGO 4 respectively. RISC loading is suggested to be regulated by RISC loading complex (RLC), which consists of Dicer, TRBP, protein activator of PKR (PACT) and the core component Ago protein (Gregory et al., 2005, Lee et al., 2006, Haase et al., 2005). During RISC forming, the double-stranded miRNA duplex is separated into a functional guide strand (mature miRNA), and a passenger strand which is typically degraded. The single stranded mature miRNA is fully incorporated with active RISC and ready to perform gene regulation (Figure 4). However, some studies pointed out that the passenger strand can also be loaded with RISC and perform active miRNA function (Ghildiyal et al., 2010, Okamura et al., 2009). The exact mechanism by which the guide strand is selected is still not very clear. Current studies suggest that the guide strand selection is determined during AGO loading step, and may mainly base on the relative thermodynamic stability of the base pairs at the two ends of the duplex: the strand with relatively lower stability is typically selected as the guide strand (Khvorova et al., 2003).

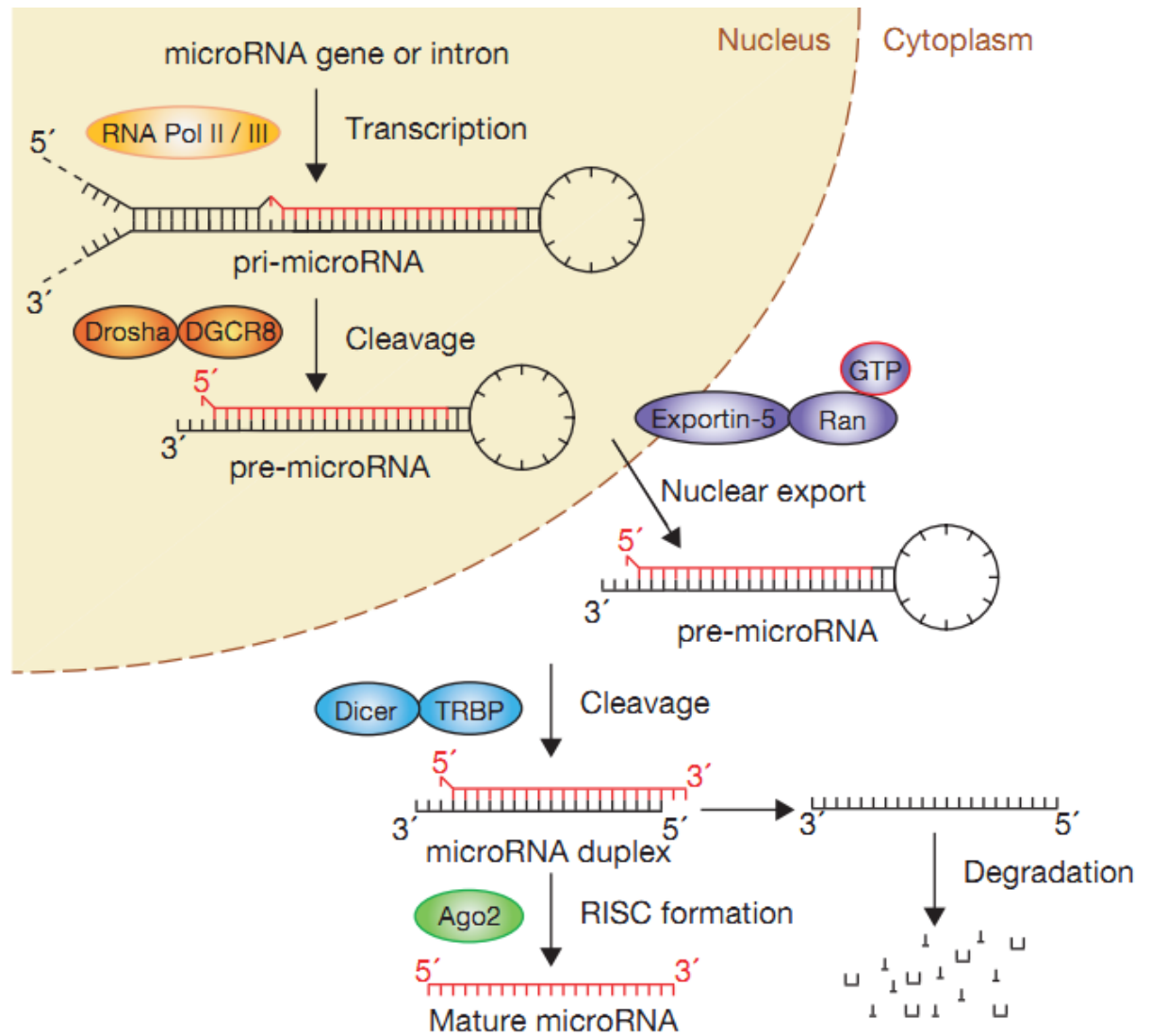


Figure 4 Biogenesis of miRNAs

miRNAs are transcribed by RNA Pol II/III into pri-miRNAs and processed into pre-miRNAs in nucleus. Pre-miRNAs are exported from nucleus and become mature miRNAs incorporated with RISC through several steps in cytoplasm (Winter et al., 2009).

1.4.2 miRNAs Nomenclature

The nomenclature of miRNAs and their genes is slightly inconsistent, especially at the time when miRNAs were initially identified in the 90s (e.g. the first known miRNAs, let-7 and lin-4). A more “standard” nomenclature scheme for miRNAs was induced later as large numbers of miRNAs were discovered (Lagos-Quintana et al., 2001, Griffiths-Jones, 2006, AMBROS et al., 2003, Lee and Ambros, 2001). Briefly, mature miRNAs are named using prefix “miR” followed by a dash and unique identify number (e.g. miR-22). miRNA genes and precursors are also named using “prefix-dash-identify number” nomenclature system, except that “mir” is used instead of “miR” (e.g mir-22). The identify numbers are assigned sequentially, which indicates that a miRNA with higher identify number is likely to be discovered later than that with lower identify number. Although the “miR/mir - xxx” system is commonly used in studies, under some circumstances another 3 letters abbreviation is prefixed to indicate the organism, for example hsa-miR-xxx means that it is mature miRNA observed from human, and in the other hand mmu-miR-xxx stands for mouse miRNAs. However, identical miRNAs have the same identify number, regardless of organism. Mature miRNAs with identical structure (usually differ from 1 -2 nucleotides) have the same identify number but annotated with an additional lower case letter (e.g. miR-121a and miR-121 b are closely related miRNAs). It is possible that completely identical mature miRNAs are generated from different

pre-miRNAs and/or genetic loci, which are differed by number suffixes at the end, e.g. mir-125b-1 and mir-125b-2 can both generate mature miR-125b. In some cases if two mature miRNAs are produced from opposite arms of the same pre-miRNA precursor, they were denoted with letter suffix: -3p (from 3' arm) or 5p (from 5' arm). However, if two miRNAs are from the same miRNA precursor, the predominantly expressed miRNA is assigned with the name of mature miRNA, while the one from opposite arm is known as miRNA*. (e.g. miR-123 and miR-123*, where miR-123 expression is much higher than miR-123* in cells)

1.4.3 miRNAs regulate their target genes

Mature miRNAs cannot regulate their target genes by themselves. Instead, they are incorporated into RISC *in vivo* and act as the “seeker” to guide RISC to their target mRNAs. Each mature miRNA can recognized and bind to several different target mRNAs through Watson-Crick base pairing. In plants, miRNAs are fully or nearly perfect complementary to their target mRNAs, while animal miRNAs usually recognize partially complementary sequences (Voinnet, 2009, Filipowicz et al., 2008). Thus the target recognition for animal miRNAs is more complex (Figure 5). It has been reported that the complementary “seed” region, which contains nucleotides 2-8 from 5' end of the miRNA, is critical for miRNA target recognition and gene regulation in animals. Also the mismatches must be located in the central region of

miRNA-mRNA duplex, and target mRNA should have reasonable complementarity to the 3' half of miRNA to stabilize the interaction.(Filipowicz et al., 2008, Huntzinger and Izaurralde, 2011, Lewis et al., 2005, Grimson et al., 2007) Majority of the binding sites were identified on 3'UTR of the target mRNA, however some studies demonstrated that mature miRNAs can bind to amino acid coding sequence and regions in 5'UTR of target mRNA(Lytle et al., 2007).

The understanding of miRNAs' function has been remarkably advanced in the past few years, but the exact mechanism by which miRNAs regulate gene expression is still unclear, especially for the effect of individual miRNA on their target genes. The "classical" thinking of miRNA-target gene regulatory machinery is that miRNAs guide RISC to their target mRNA and suppress the genes expression. The repression of target gene expression induced by miRNAs is commonly divided into three processes: i. directly site-specific cleavage. ii. mRNA decay/degradation. iii. mRNA translation inhibition (Bagga et al., 2005, Giraldez et al., 2006, Filipowicz et al., 2008). Initially, most of the discovered plant miRNAs were reported to regulate target gene through mRNA cleavage as a result of perfect or nearly perfect complementary matching, and the process is suggested to go through RNA interference (RNAi)-like pathways. Unlike plants, most of the animal miRNAs imperfectly pair with their target mRNA, and suppress their target genes expression

at translation level. However, more recent studies suggest that miRNAs can also induce mRNA degradation in animals, and conversely, in plants miRNAs can lead to translation inhibition (Brodersen et al., 2008, Gu and Kay, 2010, Selbach et al., 2008, Guo et al., 2010). Furthermore, it is still not clear that whether the target genes silencing induced by miRNAs is predominantly by mRNA degradation or translation inhibition, or combination of both pathways (Huntzinger and Izaurralde, 2011). Importantly, each miRNA can potentially regulate several target genes by using different pathways, and the regulation of target genes by individual miRNA can be affected by many factors, such as species, biological environment/conditions (Ameres and Zamore, 2013).

As mentioned earlier, mature miRNAs guide RISC to the mRNAs of their target genes and suppress genes expression. RISC is a multiprotein complex and the exact constituents of RISC are still not clear. Currently the key and best characterized components of RISC are AGO proteins (Peters and Meister, 2007, Tolia and Joshua-Tor, 2007). AGO proteins not only take part in biogenesis of miRNAs, but also play critical roles in miRNA induced gene repression (Kiriakidou et al., 2007). Mammals contain four AGO proteins, namely AGO 1 to AGO 4. All of the four AGO proteins are involved in miRNA induced repression, but only AGO2 functions like RNAi to cleavage mRNA in animals. Up to now it is suggested that AGO proteins

interact with G182 proteins and cytoplasmic poly(A) binding protein (PABPC) to carry out miRNA induced gene repression (Huntzinger and Izaurralde, 2011, Filipowicz et al., 2008). However, the downstream pathways by which AGO proteins use to suppress gene expression are largely depending on specific miRNA-mRNA interaction and other factors, which require more investigation into this field.

Although majority of the reported miRNAs go through the “classical” miRNA-target gene regulatory machinery, recent reports demonstrated that miRNA can up-regulate their target genes expression under certain circumstances. Vasudevan et al. (2007) reported that human miR-369-3 can up-regulate its target gene reporter: tumor necrosis factor- α (TNF α) containing AU-rich elements (AREs) under cell cycle arrest in HEK 293 cells. Importantly, miR-369-3 can only activate TNF α -ARE reporter under cell cycle arrest, and switch back to traditional repression in growing cells. They also conducted additional experiments by using reporters targeted by Let-7 and synthetic miRNA miRcxcr4, respectively, and the results pointed out that the reporters were up-regulated by the miRNAs under cell cycle arrest. This was the first time that miRNAs are reported to switch from repression to activation of its target gene expression. In the study Vasudevan et al. (2007) concluded the essential factors that lead to such up-regulation induced by miRNAs in their experiments: First, this

up-regulation only occurred upon cell cycle arrest at G0/1, but not other cell cycle phase or in growing cells. Second, presence of AREs in 3'UTR of TNF α mRNA is required for such activation. Third, AGO2- fragile X mental retardation-related protein 1 (FXR1) complex is required. In the following study, the importance of cell cycle arrest in mRNA induced target gene activation was further confirmed (Vasudevan et al., 2008). Other laboratories also reported miRNA induced expression of target genes. Place et al. (2008) reported that miR-373 can target promoter sequences of E-cadherin and cold-shock domain-containing protein C2, and induces their gene expression in PC-3 cells. Huang et al. (2011) reported that the promoter region of Cyclin B1 (Ccnb1) mRNA contains high-complementary sites for miR-744, and expression of Ccnb 1 can be induced by miR-744 in mouse cell line. Interestingly, miRNAs target the promoter regions of their target genes in these cases, implying that the location of the binding sites for miRNAs may also affect their function. Also, miR-155 was reported to directly and indirectly enhances its target gene *TNF- α* translation in macrophages (Tili et al., 2007), and miRNAs have also been suggested to up-regulate their target genes in immature oocytes (Mortensen et al., 2011).

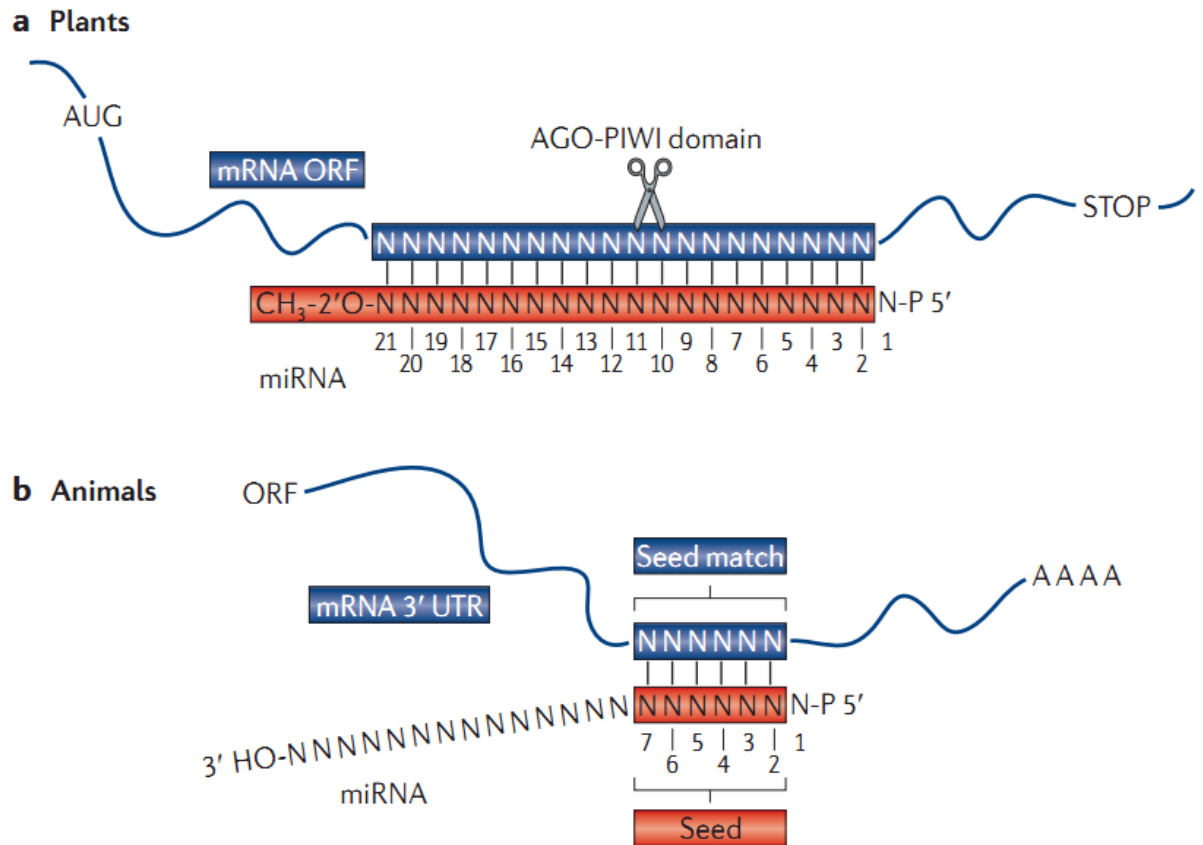


Figure 5 miRNA target recognition in plants and animals

miRNAs recognize their targets by Watson-Crick base pairing. In plants base pairing between miRNA and mRNA is perfect or nearly perfect, while animal miRNAs recognize partially complementary binding sites (Huntzinger and Izaurralde, 2011).

1.4.4 Functional role of miRNAs in cardiovascular system and ES cell differentiation

Accumulating evidences suggest that miRNAs play important roles in biological regulation of cardiovascular system development and diseases. The importance of miRNAs in such fields was first highlighted by inhibiting or disrupting miRNA

biogenesis to knockdown the “global” miRNAs level. Yang et al. (2005) generated $Dicer^{ex1/2}$ mice with two deleted essential exons for the function of Dicer, and reported that homozygous $Dicer^{ex1/2}$ died between day 12.5 and 1.45 of gestation. They also reported that from E11.5 $Dicer^{ex1/2}$ embryos displayed an impaired growth and development phenotype, and blood vessel formation was severely compromised in $Dicer^{ex1/2}$ embryos and yolk sacs. Another study carried out by Albinsson et al. (2010) pointed out that transgenic mice with deletion of Dicer in vascular smooth muscle resulted in late embryonic lethality at embryonic day 16 to 17 due to decreased SMC proliferation and differentiation, which caused thinner vessel walls, impaired contractility and hemorrhage. These data suggest that loss of miRNAs due to Dicer knockout has significant impact on embryo growth and cardiovascular system development. With the recent achievements on understanding the biogenesis and function of miRNAs, now research focus on the functional role of individual miRNA in cardiovascular system and diseases. Small et al. (2010) summarized a subset of miRNAs expressed in cardiovascular system and diseases. In the study they highlighted the important roles of miR-1, miR-21, miR-133 and miR-208a in cardiomyocyte hypertrophy, miR-195, miR-199a and miR-320 in cardiomyocyte apoptosis and regeneration, miR-21, miR-145, miR-221 in restenosis, and finally miR-221 and miR-222, miR-210, miR-126 and miR-17-92 cluster in angiogenesis. Taken together, these data clearly indicate the important roles of

miRNAs from early embryo development to regulation of mature cardiovascular system and diseases.

Stem cell differentiation plays an important role during tissue development and pluripotent stem cells hold significant potential for clinical therapies. SMC differentiation is a critical process during cardiovascular system formation and development, as well as SMC proliferative related cardiovascular diseases, such as atherosclerosis. As suggested by Landgraf et al. (2007), many miRNAs are expressed in a tissue-specific manner, implying that miRNAs may have significantly impact in cell/tissue specification. Deletion or disrupting of *dicer* or *dorsha* in ES cells lead to compromised stem cell self-renew and differentiation due to loss of “global” miRNAs, providing an overall valuation of miRNA pathways in ES cells (Murchison et al., 2005, Wang et al., 2007).

Furthermore, many recent studies have discovered the specific functional role of individual miRNA in ES cell differentiation. miR-21 is a well-known ES cell related miRNA. It has been reported that miR-21 level increased while ES cell started to differentiate (Singh et al., 2008). Further analysis showed that mRNAs of SOX2 and Nanog have potential binding sites for miR-21. SOX2 and Nanog are key factors for regulating stem cell self-renewal, and their expressions were repressed by induction

of miR-21 under stem cell maintaining condition. Furthermore, RE1-silencing transcription factor (REST), a highly expressed protein in mouse ES cells, is proved to maintain self-renewal and pluripotency of mouse ES cells through suppressing miR-21 (Singh et al., 2008). Also, miR-134, miR-296 and miR-470 were found to target mRNAs of Nanog, SOX2 and OCT4 to induce ES cell differentiation (Tay et al., 2008, Gangaraju and Lin, 2009). These findings suggest that miRNAs can directly target the key factors that controlling ES cell differentiation, and/or acting as downstream pathways of other regulators.

In additional to direct targeting the key genes which maintaining stem cell self-renewal and pluripotency, some miRNAs are also suggested to regulate stem cell differentiation in a more “complex and indirect” pathway, or even feedback loops. miR-290-295 cluster were reported to highly expressed in mouse ES cells, and controlled by a complex regulatory network consisting of Nanog, OCT4 and SOX2 (Marson et al., 2008). miR-290-295 cluster were reported to regulate OCT4 indirectly through DNA methylation. Furthermore, some studies report that miR-290-295 cluster can promote pluripotency of ES cells through regulation of cell cycle, paired-box transcription factor 6, or even promote the production of mouse iPSC (Sinkkonen et al., 2008, Lichner et al., 2011, Kaspi et al., 2013, Judson et al., 2009).

Beside the important roles of miRNAs in maintaining self-renewal and pluripotency, several studies have also suggested that miRNAs are essential players in specific SMC differentiation, and one of the best examples are miR-143/145. Human miR-143/145 cluster are located on chromosome 5q33 and regulated by a common promoter (Iio et al., 2010). miR-143/145 are suggested to be co-transcribed as a bicistronic unit, and they are direct targets of SMC transcriptional factors: SRF and myocardin, as well as Jag-1/Notch pathway (Boucher et al., 2011, Xin et al., 2009). Mice lacking of miR-143/145 have minor structural and functional defect in blood vessels but impaired neointima formation in response to vascular injury due to defect SMC migratory ability (Xin et al., 2009). More importantly, miR-145 has been proved to induce SMC differentiation from different types of stem cells by repressing their self-renewal through OCT4, SOX2 and KLF4 pathways, and increase myocardin protein through feedback from SRF-myocardin complex formation (Xin et al., 2009, Cordes et al., 2009, Xu et al., 2009). While miR-143 repress myocardin's competitor, Elk-1, to promote SMC differentiation (Wang et al., 2004, Cordes et al., 2009).

Taken together, the importance of miRNAs has been well recognized since they were found in 1993, and the understanding of miRNAs biogenesis and functions has been well advanced over the last twenty years. However, the miRNA-target gene

regulatory machinery in stem cell differentiation is still poorly defined due to the limited knowledge in the complex RISC, various downstream signal pathways involved in stem cell differentiation and specific miRNA-mRNA interaction. Further investigation into the function role of miRNAs in vascular cell differentiation from stem cells will benefit the understanding of vascular system development and disease applications.

1.5 Project Aim and objectives

SMC differentiation from stem cells plays an important role in cardiovascular system development and diseases. Better understanding of the complex molecular mechanisms underlying SMC differentiation could contribute to our knowledge of tissue development, diseases and regenerative medicine, especially in the fields of atherosclerosis, hypertension and angiogenesis. This PhD project aims to identify the potential miRNA(s) involved in SMC differentiation from ES cells and investigate the functional role of selected miRNA(s) in SMC differentiation *in vivo* and *in vitro*, as well as providing a profound study of its (their) downstream signal pathways by which mediates stem cell differentiation towards SMCs.

The objectives of this PhD project are:

1. Further validating the previous established collagen-based *in vitro* SMC differentiation model (Xiao et al., 2006) by using another ECM protein collagen type I and mouse ES cells. SMC differentiation will be assessed at RNA level and protein level. Furthermore, expression of ES cell makers and SMC transcriptional factors will also be assessed.
2. Using well established *in vitro* SMC differentiation model and the relative information collected from literatures to perform miRNA microarrays analysis. Potential miRNAs involved in SMC differentiation will be initially screened at this step, and subjected to further confirmation from *in vitro* experiment
3. Further confirm the results from miRNA microarrays analysis by checking the expression of potential miRNAs during SMC differentiation using Real-Time qPCR. Once the selected miRNA(s) is confirmed, perform gain/loss-function experiments to determine their roles in SMC differentiation from both human and mouse ES cells *in vitro*.
4. If results from *in vitro* gain/loss-function experiments are satisfied, determine whether the selected miRNA(s) has similar role in SMC differentiation *in vivo* by using Matrigel implants in mice.
5. Determine the potential target gene(s) for the selected miRNA(s)
6. Further confirm the exact target gene(s) for the selected miRNA(s). The

target mRNA(s) for selected miRNA(s) will be assessed by using gain/loss of function experiments combined with luciferase reporter assay.

7. Investigate the downstream pathways of selected miRNA(s) and its (their) target(s), including specific miRNA-mRNA interaction and their impact on SMC differentiation.

CHAPTER 2 Materials and Methods

2.1 Cell Culture

2.1.1 Making ES cell basic medium (BM)

ES cell basic medium (BM) in this project is defined as KnockOut™ D-MEM (Gibco, Invitrogen) supplemented with 100 U/ml penicillin and 100 ug/ml streptomycin. BM was stored at 4°C fridge for maximum 4 weeks,

2.1.2 Making complete ES cell culture medium (CM)

It is important to use the right culture medium for maintain ES cell to prevent differentiation. Complete ES cell culture medium was prepared into small amount (usually 50ml) and stored at 4°C fridge for maximum 8 days. To make 50ml of CM, 5ml of Foetal Bovine serum (FBS) for stem cell (Gibco, Invitrogen) was added into 45ml of BM, followed by adding 500µl of 100 × MEM Non-Essential Amino Acids Solution (MEM-NEAA, Gibco, invitrogen), 50µl Recombinant Human Leukemia Inhibitory Factor (LIF, 10µg/ml, Prospeg) and 5µl of 1M 2-Mercaptoethanol (Sigma-Aldrich).

2.1.3 Maintaining mouse ES cell

Mouse embryonic stem cells (ES-D3 cell line, CRL01934, ATCC, USA) were maintained in T25 flask with 5ml of complete ES cell culture medium (CM) in 37°C and 5% CO₂ incubator. ES cells were passaged every 2 or 3 days when reached over 70% confluency, and in a ratio of 1:6 to 1:10.

2.1.4 Passaging mouse ES cells

New T25 flask was coated with pre-warmed 0.04 % gelatin (Sigma-Aldrich) in PBS and incubated at 37° C for at least 30 minutes. After removing old medium, ES cells were washed with PBS then treated with trypsin-EDTA for 1mins in 37°C incubator. Pre-warmed CM was added into the flask to neutralize the trypsin and cells were dissociated into single cell suspension by pipetting up and down several times. ES cells were then passaged at a ratio of 1:6 to 1:10 into the pre-coated T25 flask and incubated in a 37° C, 5% CO₂ incubator for 2 to 3 days.

2.1.5 Freezing stocks of mouse ES cells

The culture medium was removed from flasks and cells were washed with PBS once, and then treated with trypsin-EDTA (Sigma-Aldrich) for 1 minute in a 37°C

incubator. BM containing 10% FBS was added into flasks to neutralize reaction, and the cell suspension was transferred into a 15ml tube and spun at $1,000 \times g$ for 5 minutes. Supernatant was then discarded and ES cells were re-suspended in freezing medium (FM) consisting of 50% DMEM (Sigma-Aldrich), 40% FBS and 10% Dimethyl sulfoxide (DMSO; Sigma-Aldrich). The cell suspension was split into cryovials a ratio of 1:4, placed into a cooling container (Mr. Frosty) and placed in a -80°C freezer overnight. Cryovials were transferred into liquid nitrogen the next day for long-term storage.

2.2 Differentiation of mESCs towards smooth muscle cells

Cell culture flasks or plates were coated with 5ug/ml collagen I/IV in cold PBS for at least 2 hours at room temperature. Undifferentiated ES cells were washed with PBS, treated with trypsin-EDTA, dissociated into single cells and seeded onto the pre-coated flasks or plates, followed by cultured in SMC differentiation medium (DM) [MEM alpha modification (Sigma-Aldrich) supplemented with 10% FBS (Gibco, Invitrogen), 0.05mM 2-mecraptoethanol, 100 U/ml penicillin and 100 ug/ml streptomycin] for 0 to 9 days prior to further treatment. The medium was refreshed every other day from day 2.

2.3 microRNA and plasmid transfection

2.3.1 Transfection of miRNA precursors or inhibitors

Transfection is the process of introducing foreign nucleic acids into cells. miRNA precursors or inhibitors and respective miRNA negative control were transfected into ES cells by using siPORT™NeoFX™ transfection agent (Ambion, Applied Biosystem) according to the manufacturer's instructions. Before preparing transfection complexes, cells were washed with PBS, dissociated into single cell with trypsin-EDTA, counted by using a hemocytometer, and re-suspended in culture medium at the correct cell density according to Table 1, and stored in 37°C and 5% CO₂ incubator while transfection complex was prepared. Warm Opti-MEM (Gibco, Invitrogen) was mixed with warm siPORT™NeoFX™ agent by pipetting and incubated for 10 minutes at room temperature, while miRNA precursors or inhibitors and their respective negative controls (10µM, Ambion, Applied Biosystems) were diluted by warm Opti-MEM in other tubes. After incubation, an equal volume of diluted siPORT™NeoFX™ agent was mixed with diluted miRNA by pipetting and incubated for another 10 minutes at room temperature to allow transfection complex to form. After final incubation, the transfection complex was add directly to pre-coated cell culture flasks or plates, followed by re-seeding the cells in culture medium as prepared above. The flaks or plates were gently tilted back and forth to evenly distribute transfection complex and cells, and incubated in 37°C and 5% CO₂

incubator. In our preliminary studies, we observed the optimized concentration of miRNA precursors or inhibitors for transfection was 30nM. Culture medium was changed 24 hours post transfection, and cells were harvested 48 hours to 72 hours after transfection for further analysis.

	T25 flask	6 well plate	24 well plate
Total number of cells	6×10^5	3×10^5	4×10^4

Table 1 Cell density used for miRNA reverse transfection

2.3.2 miR-34a and Sirt-1 siRNA co-transient transfection

Mouse ES cells were seeded on collagen coated T25 flasks in differentiation medium for 2-3 days before transfection. On the day of transfection, miR-34a mimics (MISSION® microRNA Mimic has-miR-34a, Sigma-Aldrich), SirT1 specific siRNA (Mission esiRNA targeting mouse SIRT-1, Sigma-Aldrich), or respective miRNA/siRNA negative controls (25nM each) were transfected into cells by using siPORT™NeoFX™ agent in a similar method as described in 2.3.1. Transfected cells were cultured in 5µg/ml collagen coated flasks or plates for 48 to 72 hours in the SMC differentiation medium before harvest.

2.3.3 Transfection of plasmids into ES cells

Related plasmids or respective control were transfection into cells by using Fugene-6 (Roche) or Turbofect transfection agent (Thermo) according to the manufacturer's instructions. Briefly, the ratio of transfection agent to DNA is 1:3 for Fugene-6 and 1:2 for Turbofect. On the day of transfection, normal cell culture medium was replaced by 50% volume of antibiotics free medium with 5% FBS at least 1 hour prior to transfection. Transfection agent was first diluted with serum free Opti-MEM and incubate at room temperature for 5 minutes. After incubation the diluted transfection agent was mixed with plasmids by pipetting up and down several times and incubated at room temperature for 15 to 20 minutes. After incubation the transfection complex was drop-wise added to cell culture plates or flasks with antibiotics free cell culture medium contains 5% FBS and incubated in a 37°C and 5% CO₂ incubator for 6 to 8 hours. Same volume of cell culture medium with 15% FBS was add to the cell/transfection mixtures to restore the level of FBS back to 10% and the cells were left in a 37°C and 5% CO₂ incubator overnight. On the next day the old medium was replaced by normal cell culture medium and incubated at 37°C and 5% CO₂ till 48 to 72 hours post transfection for analysis.

2.4 miR-34a overexpressing ES cells

2.4.1 Generation of miR-34a over-expression lentiviral particles

Control and miR-34a over-expression lentiviral particles were produced by using control (pLL-3.7-GFP, Addgene, Plasmid 11795) and miR-34a (pLL3.7-GFP-miR-34a, Addgene, Plasmid 25791) plasmids DNA respectively. 293T cells were cultured in T75 flask at least 24 hours prior to transfection, and then co-transfected with pLL3.7-GFP (control) or pLL3.7-GFP-miR-34a (miR-34a over-expression) and three packaging plasmids [pMDLg/pRRE (Addgene, 12251), pRsv-Rev (Addgene, 12253) and pMD2.G (Addgene, 12259)] by using Fugene-6 transfection agent. On the next day the old medium was replaced by fresh complete medium. Supernatant containing the lentivirus was collected into 15 ml tubes 48 hours post transfection and spun at 3,000 RPM for 5 minutes at room temperature. The supernatant was filtered with 0.45 μ m filters, aliquoted and stored at -80 °C for future using.

2.4.2 Lentiviral infection and cell sorting

Mouse ES cells were seeded on T25 flasks 24 hours prior to infection and incubated in 37°C and 5% CO₂ incubator. 1ml of respective lentiviral particles was added into culture medium with 10 μ g/ml polybrene (hexadimethrine bromide, H9268;

Sigma-Aldrich) and incubate at 37°C and 5% CO₂ for 24 hours. Medium containing viral constructs was replaced with complete medium after 24 hours of incubation. Cells were cultured for further 2 to 3 days and GFP-positive cells were sorted out by using anti-GFP antibody and anti-FITC microbeads (Miltenyi Biotec Ltd.) according to the manufacturer's instructions. In some cases, sorted GFP-positive cells were subjected to flow cytometry analyses to evaluate its cell purity. GFP-positive ES cells were cultured in ES cell medium for 2 to 3 passages before using.

2.4.3 SMC *in vivo* differentiation and immunofluorescent staining for sections

SMC *in vivo* differentiation was conducted as described in our previous studies (Xiao et al., 2012, Huang et al., 2013). Briefly, control or miR-34a overexpression ES cells (1×10^6 cells) were mixed with 50 µl of Matrigel (Becton Dickinson Labware) and PDGF-BB (100 ng/ml) at 4 °C and subcutaneously injected into C57BL/6J mice. After 10 to 13 days, mice were sacrificed and the implants (Matrigel plugs) were harvested. Half of each Matrigel plug was snapped frozen with liquid nitrogen and subjected to cryo-sectioned for detection of cell markers, and the rests were lysed for total RNA and protein extraction to examine related gene expression levels by Real-Time PCR and Western blot, respectively. All animal experiments were carried out according to protocols approved by the Institutional Committee for Use and Care

of Laboratory Animals.

For immunofluorescent staining, sections were cut at 8 μm for optimum cutting temperature compound-embedded Matrigel implants, every 40 μm along the longitudinal axis of Matrigel plugs. The cut sections were numbered and given numbered sections (for instance, sections 5, 15, and 25) were picked up on Superfrost Microscope Slide (Thermo Scientific) subjected to immunohistological analysis with respective antibodies. Frozen sections were air-dried for at least 30 minutes followed by fixed in cold acetone for 15 minutes. The sections were then washed with PBS and blocked with 5% BSA (Sigma-Aldrich) in PBS for 1 hour at room temperature in a humid chamber. After 1 hour the samples were incubated with primary antibodies against SM-MHC, GFP or IgG controls in blocking buffer in a cold room (4 °C) overnight. On the next day the samples were washed with PBS then incubated with appropriate FITC or TRITC conjugated secondary antibodies for 60 minutes at room temperature. After incubation the samples were washed with PBS. Finally the samples were incubated with DAPI (1:1,000, Sigma-Aldrich) for 5 minutes, washed with PBS then mounted with glycerol-PBS. Images were assessed with Axioplan 2 imaging microscope with Plan-NEOFLUAR 20X, NA 0.5 objective lenses, AxioCam camera and Axiovision software (Carl Zeiss MicroImaging, Inc) at room temperature, and processed with Photoshop software (Adobe). The percentage

of GFP-labeled SM-MHC positive cells per field was counted by two trained independent investigators blinded to the treatment from random high power fields (200×) in each sections, 3 sections from each implant and four implants from each group.

2.5 SIRT1 inhibitor and SIRT1 agonist treatment

2.5.1 SIRT1 inhibitor treatment

SIRT1 inhibitor (SIRT1 inhibitor III, EMD Millipore) were diluted in DMSO and stored in aliquots at – 20 °C. Control (pLL3.7-GFP) and miR-34a over-expressing (pLL3.7-GFP-miR-34a) were seeded on collagen coated T25 flasks in differentiation medium for 4 days to induce differentiation. On day 4, SIRT1 inhibitor (500 nm) or same volume of carrier vehicle (DMSO) were added into fresh differentiation medium and incubated at 37°C and 5% CO₂ for 6 or 12 hours before harvest.

2.5.2 SIRT1 agonist treatment

Resveratrol (Sigma-Aldrich) were diluted in ethanol at 100mM and stored at -20°C. Mouse ES cells were seeded on collagen coated flasks or plates in differentiation medium to allow SMC differentiation before treatment. Day 2-3 differentiating ES cells were incubated with fresh medium containing resveratrol (5 or 10 µm) or same

volume of carrier vehicle (ethanol) for 24 to 28 hours before harvest.

2.6 RNA extraction and analysis

2.6.1 Extraction of total RNA from cells

Before harvesting, cell scrapers were sterilized in 70% Ethanol for 10 minutes then rinsed in cold 1X PBS. Cell culture medium from flasks or plates was discarded and washed with 1 X PBS once. Cells were scrapped in cold PBS to minimum enzyme activity. Scrapped cells in cold PBS were collected in sterilized 1.5 ml tubes and centrifuged at 4°C, 6,000× g for 2 minutes. Supernatant was discarded and cell pellet were either proceed to RNA extraction or stored at - 80 °C.

All RNA related work was performed in a sterile and RNase free manner. Working surface was cleaned by using 70% Ethanol. Pipette tips and tubes were autoclaved before use to minimum the risk of RNA degradation or contamination. Total RNA was extracted by using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacture instruction. 2-mercaptoethanol was added into Lysis Solution for that day's use at a ratio of 1: 100 to fully inactivate RNase. For each tube of cell pellet (up to 5×10^5 cells), 250µl of Lysis Solution/2-mercaptoethanol mixture was added into the tube and vortexed till all

clumps disappear. Each sample was then transferred to a GenElute™ Filtration Column placed in a 2 ml collection tube, and centrifuged at room temperature, $16,000 \times g$ for 2 minutes. Filtration Column was discarded from collection tube, and 250 μ l 70% Ethanol was added to the collected supernatant then mixed well by pipetting up and down to precipitate the RNA. Up to 500 μ l of lysate/ethanol mixture from each sample was transferred into a GenElute™ Binding Column placed in a 2 ml receiving tube and centrifuged at $16,000 \times g$ for 15 seconds. After discarding the flow-through, GenElute™ Binding Column was placed back into the receiving tube. 500 μ l of Wash Solution 1 was added to each column and spun at $16,000 \times g$ for 15 seconds. The binding Column was transferred to a new collection tube. 500 μ l Wash Solution 2 was added to each tube and centrifuged at $16,000 \times g$ for 15 seconds. After discarding flow-through sample was washed again with 500 μ l Wash Solution 2 and spun at $16,000 \times g$ for 2 minutes. Wash Solution was removed from collection tube and samples were centrifuged again at $16,000 \times g$ for 1 minute to fully remove ethanol. Binding column was transferred to a new RNase free collection tube. 50 μ l of elution solution was added to each column and incubate for 1 minutes at room temperature. Samples were spun at $16,000 \times g$ for 1 minute and RNA samples were placed on ice after centrifugation. The concentration of RNA samples was then measured with Nanodrop spectrophotometers and RNA samples were stored at -80°C .

2.6.2 MicroRNA extraction

Total RNAs includes small RNAs were isolated from cells by using TRI Reagent[®](Sigma-Aldrich) according to manufacturer's instructions. Cells were scrapped from flasks in cold 1X PBS into RNase free tubes as described in 2.6.1.. 1ml of TRI Reagent[®](Sigma-Aldrich) was added to each tube of cell pellet and mixed by pipetting up and down. For cells cultured in 6-well plates, 1ml of TRI Reagent[®] was directly added to each well and the cell lysate was pipetted through a pipette tip several times to form a homogenous lysate, and transferred to RNase free tube. The samples were left at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform (Sigma-Aldrich) was added to each tube and shook vigorously for 15 seconds. The mixture was left at room temperature for 5 minutes and then centrifuged at $12,000 \times g$ for 15 minutes at 4 °C. Only colorless upper aqueous phase was transferred to a new RNase free tube, and 0.5 ml of 2-propanol was added to each tube. Samples were left at room temperature for 10 minutes then centrifuged at $12,000 \times g$ for 10 minutes at 4 °C. The supernatant was removed and RNA pellet was washed by using 1 ml of 75% ethanol and vortexed. The samples were centrifuged at $7,500 \times g$ for 5 minutes at 4 °C and the supernatant was discarded. RNA pellet was air dried for 10 minutes. 30 μ l of RNase free water was added to each RNA pellet and mixed by

pipetting. The RNA samples were then incubated at 55 °C for 10 minutes to facilitate dissolution and placed on ice, while the concentration of RNA samples was measured by Nanodrop. The RNA samples were stored at – 80 °C

2.6.3 Reverse Transcription-Polymerase Chain Reaction

Prior to Reverse Transcription-Polymerase Chain Reaction (RT-PCR) the RNA samples (excluding small RNAs) were mixed by vortex. 1 µg of RNA was diluted with RNase-free water to make a total volume of 9 µl and mixed with 1 µl of Random Primer (50ng/µl, Promega). The samples were placed on a Thermal Cycler (Techne, Staffordshire, UK) , which ran a program of 70 °C for 5 minutes followed by cooling down to 4 °C to destabilize RNA secondary structures and enhance primer binding. The master mix for RT-PCR was prepared while the program was running. The volume of master mix for each reaction is 15 µl, which consists of 4.5 µl of RNase-free water, 5 µl of 5X Reaction buffer (Promega), 3 µl of 25mM magnesium chloride (MgCl₂), 1 µl of 25mM 2'-deoxynucleoside 5'-triphosphate (DNTPS; Invitrogen), 1 µl of Improm-II reverse transcriptase and 0.5 µl of RNasin Plus RNase inhibitor (Promega). The master mix was combined with samples and placed into the block of Thermal Cycler, which ran a program of 25 °C for 5 minutes for annealing followed by an extension at 42 °C for 90 minutes. The reaction was then stopped by enzyme denaturation at 70 °C for 15 minutes, then cooled down and

hold at 4°C. The final concentration of each synthesized complementary Deoxyribonucleic acid (cDNA) samples was diluted to 5ng/μl and stored at -20 °C.

NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen) was used to synthesize poly(A) tails of all the microRNA, and then synthesizing cDNA from the tailed population in a single reaction. Each reaction consists of 500 ng RNAs, 2 μl of 5× Reaction Mix, 1 μl of 10× SuperScript® and topped up to 10 μl with RNase-Free water. The samples were vortexed to mix and centrifuged briefly before placing in a Thermal Cycler, which ran a program set at 37 °C for 60 minutes then raised to 95 °C for 5 minutes to terminate the reaction, and held at 4 °C at the end. The cDNA samples were diluted to 5ng/ μl and stored at -20 °C.

In some experiments, negative control or mock reverse transcription containing all the RT reagents, except the reverse transcriptase (for normal RNA) or 10× SuperScript® (for miRNA), respectively, were conducted to distinguish genomic DNA contamination.

2.6.4 Real-Time Polymerase Chain Reaction

The working area was cleaned with 70% Ethanol and only sterilized pipette tips and

tubes were used when preparing Real-Time Polymerase Chain Reaction (RealTime-PCR) samples. cDNA samples were thawed from -20 °C and placed on ice. The master mix for one reaction of RT-PCR for mRNA contained 5 µl of KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems), 0.75 µl of 2 µM forward primer, 0.75 µl of 2 µM reverse primer and 1.5 µl of RNase-free water. For microRNA samples, the master mix consists of 5 µl of NCode™ EXPRESS SYBR® GreenER™ qPCR SuperMix, 0.75 µl of 2 µM miRNA specific forward primer, 0.75 µl of 2 µM miRNA Universal Primer (Invitrogen) and 1.5 µl of RNase-free water. The master mix was vortexed then aliquoted at 8 µl for each reaction in a well of a MicroAmp® Optical 384 wells plate. 2 µl of 5 ng/µl cDNA sample was added into its respective well and each sample was carried out in duplicates. The 384 wells plate was sealed with MicroAmp® PCR film, vortexed briefly and centrifuged at $1,200 \times g$ for 1 minute. ABI HT 7900 was used to run the PCR reaction, which has a program of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95 °C for 15 seconds and 60°C for 1 minute. Once the reaction has completed, the result was analyzed with SDS 2.3 software (Applied Biosystems). The Pfaffl's method (Pfaffl, 2001) was applied to determine the different expression levels of a gene of interest between a control and a treated sample. Briefly, the amount of the target gene is normalized towards an internal control housekeeping gene (in this case the 18S gene or U6 RNA) in both the control and the treated sample and it is expressed by the

formula: $2^{-\delta\delta Ct}$, where $\delta\delta Ct = \delta Ct,T - \delta Ct,C$, with $\delta Ct,T$ as the difference between the Ct of the gene of interested and the housekeeping gene in the treated sample and $\delta Ct,C$ as difference between the Ct of the gene of interested and the housekeeping gene in the control sample.

2.7 Protein extraction and analysis.

2.7.1 Chemicals and buffer

Name	Components
RIPA buffer	Tris-HCl: 50mM, pH 7.4. NP-40:1% Na-deoxycholate: 0.25% NaCl: 150mM
5× SDS loading buffer	Tris-Cl (0.25 M, pH 6.8) SDS (sodium dodecyl sulfate; 10%) Glycerol (50%) Bromophenol blue (0.25%) DTT (dithiothreitol; 0.5 M)

10× Tris Glycine buffer, pH8.4 1 Liter	Tris base: 30.3g Glycine:144.1g Distilled water to 1 Liter
1× Running buffer 1 Liter	10 × Tris Glycine buffer: 100 ml 10% SDS: 10ml Distilled water to 1 Liter
1 × Transfer buffer 1 Liter	10 × Tris Glycine buffer: 100 ml Methanol : 200ml Distilled water to 1 Liter
10 × TBS buffer 1 Liter	Tris HCl: 24g Tris Base:5.6g NaCl: 88g Distilled water to 1 Liter
1 × TBS-T	10 × TBS: 100ml Tween® 20:500µl Distilled water to 1 Liter

Table 2 Recipes of buffer used in protein analysis

All chemicals were from Sigma Aldrich unless specifically indicated.

2.7.2 Protein extraction from cells

Cell pellets were collected by using cell scrappers in cold 1× PBS. After removing of PBS, the cell pellets were either stored at - 80°C or proceed to protein extraction. Each cell pellet was mixed with 100 µl of Lysis buffer [RIPA buffer containing Protease Inhibitor Cocktail (Sigma-Aldrich)] and sonicated at 4°C to disrupt cell membrane and release protein contents. The lysates were left on ice for 1 hour and vortexed every 15 minutes to allow fully lysis of cells. After incubation the samples were centrifuged at 16,000 × g for 10 minutes at 4 °C. Up to 100 µl of supernatant from each sample was transferred into new pre-cooled 0.5 ml tubes and placed on ice. The concentrations of proteins were measured by using BIORAD Protean assay solution (BIO-RAD, Herts, UK). The assay solution was first diluted at 1:5 by using Distilled water. 2 µl of each protein sample was added to 998 µl of diluted BIORAD Protean assay solution, vortexed and incubated for 10 minutes at room temperature. Protein concentrations were measured by using a spectrophotometer (SmartSpec™ 3000; BIO-RAD). Lysis buffer and warmed 5× sodium dodecyl sulfate (SDS) protein loading buffer were then added into each sample to adjust the concentration. The

protein samples were placed into a heat block and denatured at 95°C for 10 minutes, followed by vortexing and then centrifuged briefly. After cooled down at room temperature for 3 minutes, the samples were stored at – 80 °C.

2.7.3 Western blot and immunodetection

Electrophoresis was carried out with Mini-PROTEAN® Tetra Cell system (BIO-RAD, UK) and the protein was separated by SDS PAGE with 6-10% Tris-clycine gel. The gels were prepared on the day of experiment, and 4ml of 10% separating gel consists of 1.6ml double-distilled water, 1.635ml of 30% Acrylamide (National diagnostics, USA), 1ml of 1.5M Tris buffer pH 8.8, 40µl of 10% SDS, 40µl of 10% APS and 4 µl of TEMED, while 2.5 ml of 6% Stacking gel consists of 1.3 ml of double-distilled water, 0.5 ml of 30% Acrylamide, 0.625 ml of 0.5M Tris buffer pH 6.8, 25 µl of 10% SDS, 25µl of 10% APS and 2.5 µl of TEMED. 4ml of separating gel was first added into the glass casting chamber followed by 1ml of butanol (Sigma-Aldrich) to flatten the gel. After 10 minutes, the butanol was discarded from the chamber and 2.5 ml of stacking gel was added on top of the separating gel. Plastic comb was placed into the gel to create 10 wells in each gel, and incubated at room temperature for 10 minutes. 40 µg of each protein sample was added into the wells of the gel, with 15 µl of ColorPlus Prestained Protein Ladder (New ENGLAND BioLabs®inc) loaded in the first well of the gel. The gels were run

at a fix voltage of 160 V for 1 hour 15 minutes in 1× running buffer at room temperature. Polyvinylidene difluoride (PVDF) membranes (GE Healthcare) were first activated in methanol (Sigma-Aldrich) then soaked in 1× transferring buffer. Blotting sponges were washed with distilled water then soaked in 1 × transfer buffer with filter papers. The gels were transferred into blotting cassettes with pre-wetted membranes, blotting sponges and filter papers. The assembly was carried out in 1× transfer buffer and air bubbles were removed by rolling over the surface with a roller. The blotting cassettes were inserted into a Mini Trans-Blot Central Core (BIORAD) and placed back to the running cell filled with cold 1× transfer buffer and a cooling pack. The proteins were electro-transferred to membranes for 2 hours 30 minutes at 60 V.

The membranes were removed from blotting cassettes after transfer and blocked in 5% silk milk in TBST buffer on a 2-D rocker, with the protein facing upwards for 1 hour at room temperature. The membranes were then incubated with primary antibodies in 5% silk milk in TBST on a slow roller at 4 °C overnight.

The membranes were taken out from primary antibodies and washed with 1× TBST 3 times for 10 minutes each wash on a shaker, and then incubated with horseradish peroxidase conjugated secondary antibodies in 5% milk TBST for 1 hour at room

temperature. After incubation, the membranes were washed again with 1× TBST 3 times for 10 minutes each wash. Finally the membranes were incubated with ECL-PLUS reagent (Amersham Biosciences, Stockholm, Sweden) for 3 minutes at room temperature. After removing the excess ECL reagent, the membranes were wrapped in a clean plastic film and placed in an autoradiographic cassette. Membranes were exposed to Hyperfilm ECL (GE Healthcare) for normally 10 seconds to 2 minutes (depending on signal strength) in a dark room, and films were developed with an X-Ray film processor (SRX-101A. Konica Minolta, USA).

2.8 Transient transfection and luciferase assay

2.8.1 SirT1 3'UTR reporter cloning and miR-34a binding sites mutation

Reporter vector harboring sequences of murine SirT1 was created using cDNA from differentiating ES cells. The 3'-flanking untranslation region (2306bp/3636bp) of murine SirT1 gene (NM_019812.2) containing two miR-34a binding sites was amplified by PCR with primers shown in appendix, and cloned into pmiR-reporter-basic vector (Ambion, Applied Biosystem) at Mlu I and Sac I sites, designated as pmiR-Luc-SirT1-WT. miR-34a binding sites 1,2 mutation alone or

dual mutation were introduced into pmiR-Luc-SirT1-WT by using QuickChange™ site-directed mutagenesis kit (Agilent Technologies) according to manufacturer's instructions. They were designated as pmiR-Luc-SirT1-BS1^{mu}, pmiR-Luc-SirT1-BS2^{mu} and pmiR-Luc-SirT1-BS1/2^{mu} mutants, respectively. An artificial miR-34a binding site from SirT1 3'UTR was introduced into pmiR-Luc by using PCR based on QuickChange™ site-directed mutagenesis, designated as pmiR-Luc-miR-34a binding site. All vectors were verified by DNA sequencing from Genome Centre, WHRI.

2.8.2 Amplification and extract of plasmid

Transformation was used to amplify DNA. Tubes and pipette tips were sterilized before use and working area were sterilized with 70% Ethanol. Competent cells (JM109) were taken out from -80°C and thaw on ice. The cells were aliquoted into cold 0.5ml tubes (20µl/tube) and mixed with 1 µl of plasmids/DNA or negative control (DDH₂O). The mixture was incubated on ice for 30 minutes then placed into a heat block at 42°C for 60 to 70 seconds, followed by incubating on ice for 1 minute. 500µl of LB medium was added into each tube and the samples were incubated at 180 RPM, 37 °C in a floor shaker for 1 hour. The mixture was then centrifuged at 5,000 x g for 5 minutes, and 400 to 450 µl of supernatant was discarded. The cells were re-suspended in the remaining supernatant and added into LB-plates drop-wise

with appropriate antibiotics by pipetting. The LB-plates were lightly smashed with sterilized “L” rode and placed into a 37 °C incubator to culture overnight. On the next day, single clone was taken into 14ml tubes containing 5ml LB with antibiotics and placed into a floor shaker at 200 RPM, 37 °C to culture for 12 to 18 hours. The samples were centrifuged at $4,000 \times g$ for 10 minutes and supernatant was discarded to collect the cell pellets. Plasmids were extracted using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to manufacturer’s instruction. The concentrations of plasmid were measured using NanoDrop and plasmids were stored at – 20 °C.

2.8.3 Luciferase activity assay

Undifferentiated, day 2-4 differentiating ES cells or terminally differentiated (Day 8) SMCs (40,000 cells per well of 24-well plate) were co-transfected with individual reporter gene (pmiR-Luc-SirT1-WT, pmiR-Luc-SirT1- BS1^{mu} , pmiR-Luc-SirT1-BS2^{mu}, pmiR-Luc-SirT1-BS1/2^{mu}, pmiR-Luc-ZEB1 (Luo et al., 2013) , pmiR-Luc-MECP2 or pmiR-Luc-miR-34a binding site, 180 ng/well), pRenilla (20 ng/well), and mir-34a precursor and negative control miRNA precursor (30nM) by using siPORT™NeoFX™ according to the manufacturer’s instructions. The cells were cultured on gelatin or collagen coated 24-well plate for 48 hours after transfection, with 1 medium change at 24 hours post transfection.

After 48 hours of incubation, culture medium was removed from wells and the cells were washed with 1× PBS once. After removing of PBS, 100µl of Reporter Lysis Buffer (Promega) was added into each well and the cell culture plate was placed on a 2-D rocker at 200RPM for 20 minutes at room temperature. The cell culture plate with Lysis buffer was placed into a -80°C freezer for at least 2 hours and thaw on a 2-D rocker again at 200 RPM for 20 minutes at room temperature. The lysates from each well were transferred into 1.5ml tubes and centrifuged at 16,000 x g at 4 °C for 5minutes. 15 µl of supernatant from each samples were mixed with Luciferase or Renilla substrates (Promega) to detect the activities with a Single-Tube Luminometer (Turner BioSystem, Sunnyvale, CA, USA). Relative Luciferase unit (RLU) was defined as the ratio of Luciferase versus Renilla activities with that of the control set as 1.0.

For Gene promoter luciferase activity assays, day 2 – day 4 differentiating cells cultured in 24-well plate (40,000 cells) were co-transfected with respective gene promoter reporter (pGL-3-Luc-gene, 150 ng/well), *pshuttle2-LacZ* (200 ng/well) or control plasmid (pcDNA 3.1, 200 ng/well), or SirT1 overexpression plasmids (pcDNA3.1-SirT1, 200 ng/well) using Fugene-6 (Roche) or Turbofect transfection agent (Thermo) according to the manufacturer's instructions. The cells were cultured

in differentiation medium for 48 to 72 hours after transfection with 1 medium change at 24 hours post transfection. The gene promoter reporters used in this study were generated in our previous studies (Huang et al., 2013, Margariti et al., 2009), including pGL3-Luc-Sm α A, pGL3-Luc-SM22 α , pGL3-Luc-SM α A-SRF^{mu}, pGL3-Luc-SM22 α -SRF^{mu}, pGL3-Luc-SRF pGL3-Luc-MEF2c and pGL3-Luc-Myocardin. pShuttle2-LacZ (0.20 μ g/ 2.5×10^4 cells) was included in all transfection assays as internal control. Luciferase activity was detected as described above and β -galactosidase activity was measured using a standard protocol. Relative Luciferase Unit (RLU) was defined as the ratio of Luciferase versus β -galactosidase activities with that of the control set as 1.0.

2.9 Immunoprecipitation

2.9.1 Chromatin immunoprecipitation (ChIP) assay

Differentiating ES cells were transfected with control (pcDNA3.1) or SirT1 over-expression (pcDNA3.1-SirT1) plasmids using Turbofect transfection agent (Thermo) in T75 flasks and cultured for 48 hours after transfection, with one medium change at 24 hours. 1% (v/v) of formaldehyde was added into cell culture medium in T75 flasks, incubated at room temperature for 10 minutes and then quenched with

glycine. The medium was removed and cells were harvested, resuspended in SDS lysis buffer and sonicated. The sheared samples were diluted into 1ml of immunoprecipitation buffer containing 25mM Tris-HCL, pH 7.2, 0.1% NP-40, 150 mM NaCl, 1mM EDTA, and immunoprecipitation were carried out with antibodies (2µg/immunoprecipitation) raised against SirT1 (rabbit, H-300, sc-15404, Santa Cruz), SRF (rabbit, G-20, sc-335, Santa Cruz) or H3K9me3 (mouse, 05-1250, Millipore) together with single-strand salmon sperm DNA saturated with protein-G-Sepharose beads. Equal amount of normal rabbit IgG or mouse IgG was used as control. The samples were placed on a roller and incubated at 4 °C overnight. The immunoprecipitations were eluted from the beads using 100 µl elution buffer (50mM NaHCO₃, 1% SDS). 200 µl of proteinase K solution was added to each samples and incubated in a heat block at 60 °C overnight. Immunoprecipitated DNA was extracted, purified and then used to amplify target DNA sequences by Real-Time PCR with specific primers. Relative DNA level (or promoter DNA enrichment) was defined as the ratio of immunoprecipitated promoter DNA level to its input level with that of the control sample (pcDNA3.1) set as 1.0. PCR amplification of the adjacent promoter regions or regions lacking of SRF binding sites (without CArG region) were included as additional control for specific promoter DNA enrichment. The respective target promoter DNA was almost undetectable or with much higher CT value in normal IgG control samples. The data was obtained from four independent

experiments.

2.9.2 RNA immunoprecipitation (RNA-IP) assays

Undifferentiated ES cells or day 4 to day 6 differentiating SMCs were treated with 1% formaldehyde at room temperature for 10 minutes and then quenched with glycine at room temperature. Cells were harvested, resuspended in Lysis buffer containing RNase inhibitor, and sonicated in a sonicate machine (SpishaeTM Probe Sonicator). The sheared samples were diluted into 1ml immunoprecipitation buffer and incubated with 5µg of Ago 2 antibody (Fisher Scientific, MA5-14861) or equal amount of rabbit IgG together with protein-G Dynabeads® saturated with single-strand salmon sperm DNA at 4 °C overnight. The immunoprecipitates were saturated with DNase and eluted from the beads using 100 µl of elution buffer. The immunoprecipitated RNA was extracted, purified and then used to synthesize cDNA by using NCodeTM VILOTM miRNA cDNA Synthesis Kit according to manufacturer's instructions. The cDNA samples were used to amplify target RNA sequences by Real-Time PCR using specific primers. The respective target RNA/miRNA levels were almost undetectable or with much higher CT value in normal IgG control samples. RNA enrichment with specific antibody was calculated using percent input method with that of the IgG control set as 1.0. The relative level of RNA enrichment was defined as the ratio of RNA enrichments in the treatment

groups (differentiated SMCs) to the control groups (undifferentiated ES cells) with that of the control samples set as 1.0. PCR amplification of the murine SirT1 coding region was included as additional control for specific RNA enrichment.

2.10 Flow cytometry, cell cycle, proliferation, and apoptosis analysis.

2.10.1 Flow cytometry analysis

Flow cytometry analysis were performed either using direct or indirect method depending on the antibodies of interest. Differentiated ES cells were dissociated into single cells by trypsin-EDTA, counted in culture medium and centrifuged at 1,000 \times g for 5 minutes. The cell pellets were collected and washed with cold 1 \times PBS containing 10% FBS. 1 ml of cold 4% Paraformaldehyde (PFA, for per 1 \times 10⁵ to 5 \times 10⁵ cells) was mixed with cells and incubated on ice for 15 minutes. The mixture was then centrifuged and supernatant was removed. For intracellular markers detection, 1 ml of cold permeabilization buffer (PBS-0.1% Triton X-100, per 1 \times 10⁵ to 5 \times 10⁵ cells) was added to the cell pellet, vortexed and incubated on ice for 5 to 10 minutes. Permeabilization buffer was removed by centrifuged and the cell pellets were resuspended in 10 % FBS in PBS to adjust the cell concentration to 1 \times 10⁷ per ml, and incubated on ice for 20 minutes to block non-specific antibody

binding. 100 μ l of single cell suspension was aliquoted into individual tubes and stained with either antibodies against GFP, SM α A, SM-MHC, or Isotype IgG negative controls. The mixture was vortexed and incubated for 1 hour at room temperature in a dark box. After 1 hour, each tube was washed with 1 ml of cold PBS and supernatant was removed by centrifugation and pipetting. Appropriate amount of secondary antibodies (1:50 to 1:100) was diluted with PBS containing 10% FBS and mixed with the respective cell pellets. The mixture was incubated at room temperature in a dark box for 30 minutes and then washed twice with cold PBS. After removing of PBS, the cell pellets were re-suspended in 400 μ l of 1% PFA and analyzed using a FACSCalibur sorting system (Becton Dickinson).

2.10.2 Cell cycle and apoptosis analysis

Mouse ES cells at the indicated time points or with different treatments were dissociated into single cells using trypsin-EDTA and collected in cell pellets. The cell pellets were washed with PBS twice and then re-suspended in 300 μ l of PBS. 3 ml of 70% ethanol was added drop-wise into the cell pellets and incubated at 4 °C for at least 30 minutes. Fix cells were then washed twice with PBS, and incubated in PBS containing 0.01 % Triton X-100, 40 μ g/ml RNase and 50 μ g/ml propidium iodide (All from Sigma-Aldrich) for 45 minutes at 37 °C. The samples were washed again with PBS the re-suspended in PBS and cell cycle distribution was analyzed using a

FACSCalibur sorting system (Becton Dickinson).

For apoptosis analysis, day 2 to day 3 differentiating ES cells were transfected with miR-34a precursor or negative control and cultured in differentiation medium for 48 hours after transfection. The cell pellets were collected and subjected to apoptosis analysis using Annexin V-FITC/PI kit (BMS306F1; Bender MedSystem) according to manufacturer's instructions. The binding buffer was first diluted at 1:4 in distilled water. The cell pellets were washed with PBS and resuspended in the diluted binding buffer to adjust the cell density to $2 \sim 5 \times 10^5$ per ml. 195 μ l of cell suspension was mixed with 5 μ l of Annexin V-FITC and incubated for 10 minutes at room temperature. After incubation the samples were washed with PBS once, centrifuged and re-suspended in 190 μ l of diluted binding buffer. 10 μ l of 20 μ g/ml Propidium iodide stock solution was added into each sample and cell apoptosis was analyzed with FACSCalibur sorting system.

2.10.3 BrdU incorporation

Cell proliferation was evaluated using 5-bromo-2'-deoxyuridine (BrdU) Labeling and Detection Kit III (Roche) according to the manufacturer's instructions. Day 2 to day 3 differentiating ES cells were transfected with miR-34a precursor or negative control and seeded in gelatin coated 96-well plates (5×10^3 cells per well) for 18

hours. Cells were then incubated with fresh differentiation medium containing 10 μ M BrdU for 12 hours. After removal of culture medium, the cells were washed with wash medium containing 10% FBS and then fixed with 200 μ l of precooled fixative for 30 minutes at -20°C . The fixed cells were washed with 10% FBS wash medium, and cellular DNA was digested with nuclease by incubating with 100 μ l of nucleases working solution per well for 30 minutes at 37°C . The samples were washed with washing buffer containing 10 % FBS and labeled with peroxidase-conjugated anti BrdU antibody (100 μ l/well) for 30 minutes at 37°C , followed by washing 3 times with wash solution II and then incubated with peroxidase substrate (100 μ l/well) at room temperature until positive samples showed a green color. The absorbance of the samples was measured by a microplate reader at 450nm with a reference wave-length at approx. 490 nm.

2.11 Materials

Antibodies against SirT1 (rabbit, H-300, sc15404), SRF (rabbit, G-20,sc-335) and MEF2c (goat, sc-13268) were from Santa Cruz Biotech, USA. Antibody against Smooth Muscle Myosin Heavy Chain (SM-MHC) was from AbD Serotec (Rabbit, AHP1117). Antibodies against SM22 α (Rabbit, AB14106) and calponin (rabbit, Ab46794) were from Abcam, UK. Antibodies against α -tubulin (mouse), SM α A (Clone 1A4, A5228) GAPDH (mouse) and GFP (G6539) were from Sigma Aldrich.

Antibody against H3K9me3 (mouse, 05-1250) was from Millipore. All secondary antibodies were from Dako, Denmark. Random siRNA control (MISSION® esiRNA, Negative Control) was from Sigma Aldrich. Other materials used in this study were from Sigma Aldrich unless specifically indicated.

Primers for PCR related work and gene cloning were listed in Appendix.

2.12 Statistical analysis

Data were presented as mean \pm standard error of the mean (SEM) and analyzed using a two- tailed student's *t*-test for two group comparison or one-way ANOVA followed by Turkey's HSD multiple comparison post-hoc test for comparing different groups. P value less than 0.05 were considered statistically significant.

CHAPTER 3 SMC differentiation induced by collagen

Pluripotent mouse ES cells can differentiate into different types of cells, including vascular SMCs. It has been reported that one of extracellular matrix (ECM) proteins, collagen type IV, promotes SMC differentiation by activating several signal pathways (Huang et al., 2013, Pepe et al., 2010, Xiao et al., 2012, Xiao et al., 2011, Xiao et al., 2007). Interestingly, in these studies another ECM protein, type I collagen has also been suggested to play a similar role in SMC differentiation. To further confirm such a possibility, mouse ES cells were cultured in collagen type I coated T25 flasks and culture in SMC differentiation medium (DM) in the absence of LIF for 2 to 8 days to allow for SMC differentiation. A set of SMC markers, as well as SMC transcription factors and undifferentiated ESC markers were analyzed using real-time PCR and western blot, respectively. Taken together, the data demonstrated an increase expression of all the SMC specific genes examined in this study and several transcription factors over 8 days of differentiation, mostly reaching maximum level at day 6, while the gene expression levels of several undifferentiated ESC-specific transcription factors were significantly decreased from day 4, further validating the collagen-based SMC differentiation model.

3.1 SMC specific genes were up-regulated during ES cell differentiation

Undifferentiated ES cells were cultured in collagen type I coated T25 flasks and cultured in differentiation medium in the absence of LIF. Cells were harvested at the indicated time points (day 0 to day 8). Total RNA was extracted and subjected to real-time PCR analysis. mRNA level of SMC specific genes, including SM α A, SM-MHC, Calponin and SM22 α was significantly up-regulated from day 4 of differentiation as compared with that of day 0 (undifferentiated ES cells) and reached maximum level at day 6 (Figure 6). In contrast, expression level of undifferentiated markers including OCT3/4, Nanog and KLF4 was significantly decreased from day 2 as compared with that of day 0 (Figure 7).

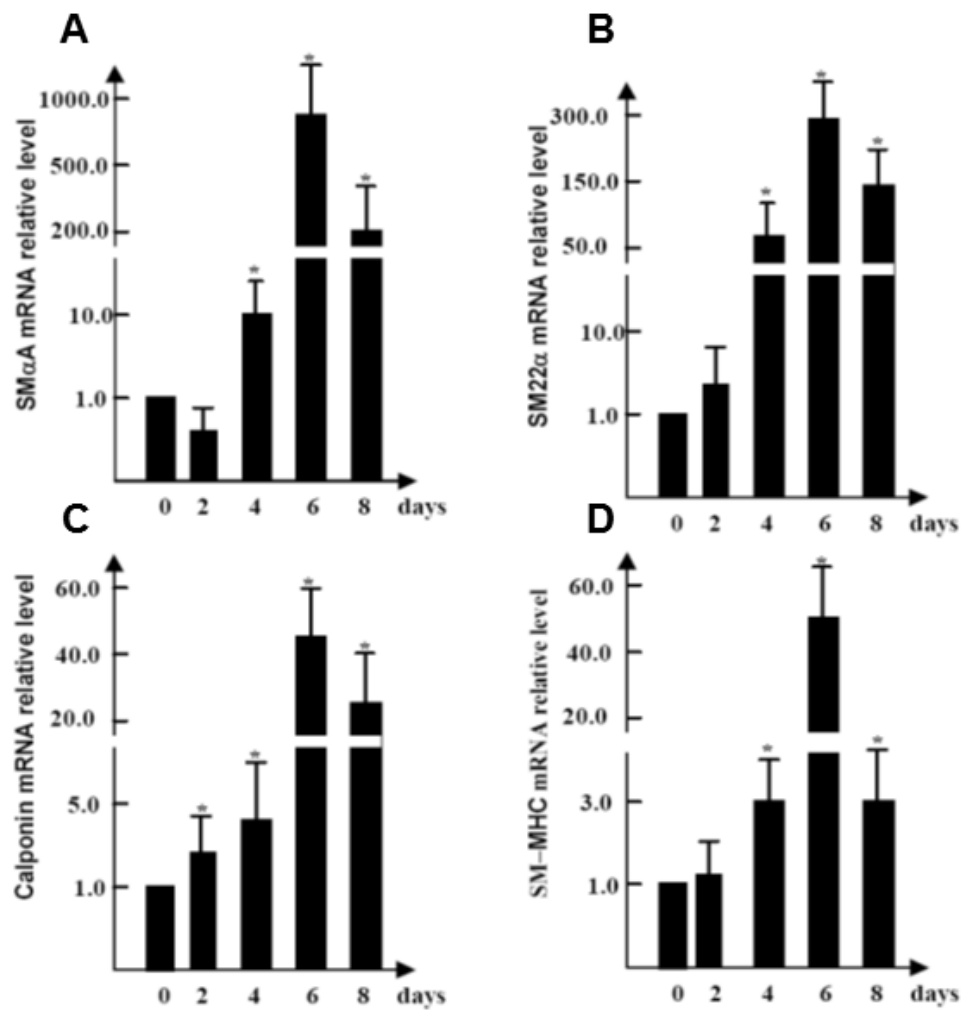


Figure 6 SMC specific gene expression over a period of 8 days

Undifferentiated ES cells were cultured on collagen type I coated flasks and cultured for 2, 4, 6 and 8 days. Day 0 represent undifferentiated ES cells. Cells were harvested at indicated time points to extracted total RNA and subjected to real-time PCR with specific primer for SM α A, SM22 α , calponin and SM-MHC. Data represent mean \pm SEM of three independent experiments (n=3). mRNA level was normalized to 18s and presented relative to day 0 expression. Significant difference from control (day 0), *P<0.05.

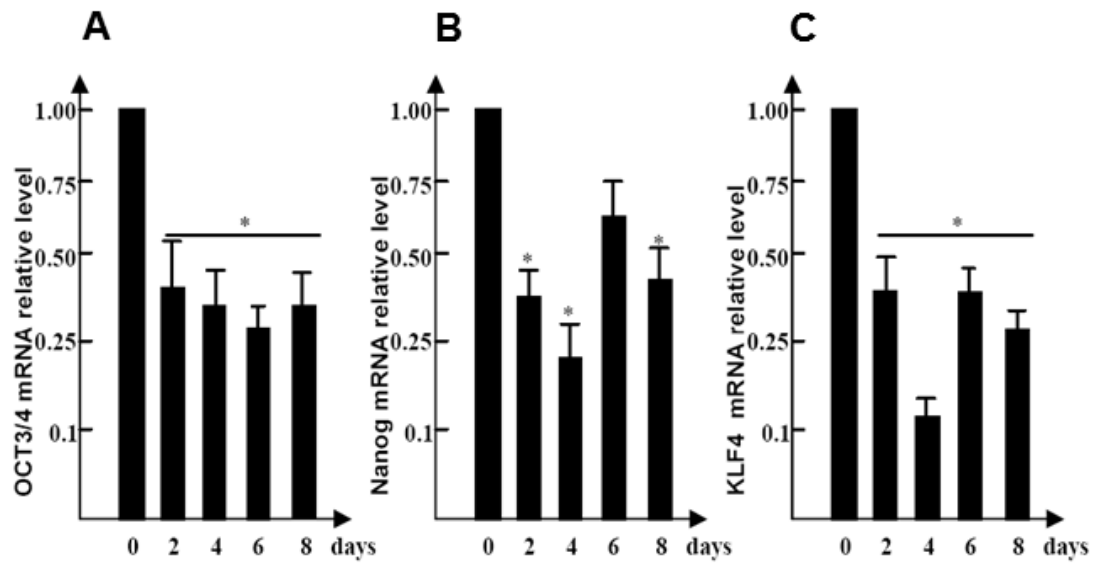


Figure 7 Stem cell specific transcription factors were silenced during SMC differentiation

Same total RNA samples were harvested as described in Figure 1 and subjected to real-time PCR with specific primer for OCT3/4, Nanog and KLF4. Data represent mean \pm SEM of three independent experiments (n=3). mRNA level was normalized to 18s and presented relative to day 0 expression. Significant difference from control (day 0), *P<0.05.

3.2 SMC transcription factors gene expressions were activated during SMC differentiation.

SRF, Myocardin and MEF2C play an important role in SMC differentiation from ES cells. To establish the expression profiles of these SMC transcription factors in the SMC differentiation method, undifferentiated ES cells were induced to differentiate into SMCs as described above and cells were harvested at indicated time points. Total RNA was extracted and subjected to Real-Time PCR analysis. The results suggest that mRNA level of SRF and MEF2C significant increased from day 4, and reached maximum level at day 6 (Figure 8A and B), while mRNA level of myocardin increased at day 6 and reached maximum value at day 8 (Figure 8C), as compared with mRNA level of respective SMC transcriptional factors from undifferentiated ES cells.

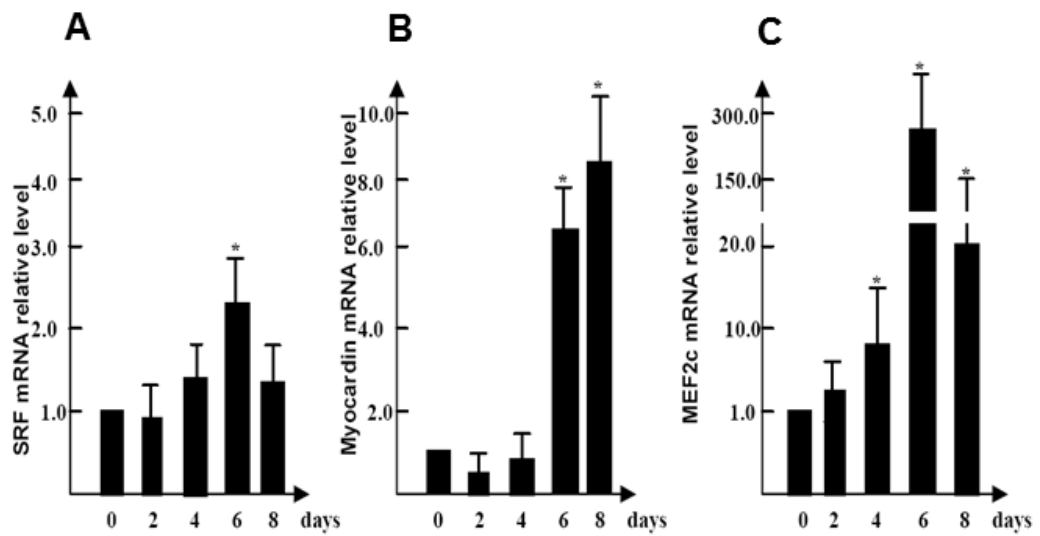


Figure 8 Expression of SMC transcription factors gene over 8 days of SMC differentiation

Same total RNA samples were harvested as described in Figure 1 and subjected to real-time PCR with specific primers for SRF (A) Myocardin (B) and MEF2C (C). Data present mean \pm SEM of 6 independent experiments. mRNA level was normalized to 18s and presented relative to day 0 expression. Significant difference from control (day 0), *P<0.05.

3.3 Western blot analysis of SMC specific marker expression in differentiating ES cells.

Western blot was carried out to determine SMC specific gene expression product at indicated time points during SMC differentiation. Strong protein expressions of both SM α A and calponin was detected from day 6, while undifferentiated ES cells (day 0) did not express any SMC specific markers (Figure 9).

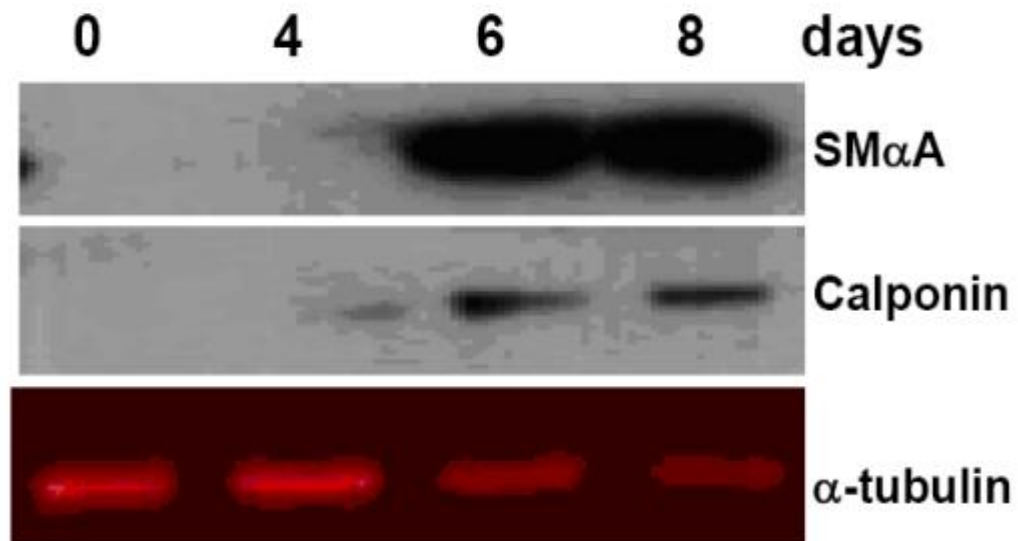


Figure 9 Western blot analysis of ES cells differentiation over 8 days.

Undifferentiated ES cells were seeded on collagen coated flasks and cultured for 2, 4, 6 and 8 days. Day 0 represented undifferentiated ES cells. The samples were detected by western blot analysis with antibodies against SM α A (Top) and calponin (Middle), respectively. α -tubulin was used as housekeeping protein (Bottom). The data presented here is representative of 3 independent experiments.

Chapter 4 Function role of miR-34a in SMC differentiation from ES cells

Data from chapter 3, as well as other studies (Huang et al., 2013, Pepe et al., 2010, Xiao et al., 2012, Xiao et al., 2011, Xiao et al., 2007) suggested that collagen based stem cell differentiation model is a simple but efficient SMC differentiation model, which provides a useful platform to investigate the potential role of individual miRNA during SMC differentiation.

4.1 Functional involvements of miR-34a in SMC differentiation *in vitro* and *in vivo*

4.1.1 Screening potential miRNA during ES cells differentiation towards SMCs

In order to identify potential miRNA candidates for SMC differentiation from ES cells, total RNA including small RNA from undifferentiated ES cells and differentiated ES cells over 4 days or 8 days were harvested and subjected to microRNA microarrays analysis (Miltényi Biotec GmbH, Germany). Data from microarrays revealed that except miR-294, all other five members of miR-290 family, which were reported to be mouse ES cells specific miRNA cluster (Houbaviy et al.,

2003), were down-regulated especially at day 8 of differentiation, further confirming cell differentiation (Table 3) SMC differentiation related miRNAs such as miR-143, miR-145 and miR-133 were increased in the SMC differentiation model, while miR-21 which involved in SMC proliferation was undetected at early stage of differentiation (day 4) but significantly increased at late stage (day 8), indicating that some miRNAs (eg. miR-143/145) may initiate SMC differentiation whereas others (eg. miR-21) may play important roles in the late stage of SMC differentiation where differentiated SMC proliferation is required to complete the SMC differentiation process. No significant changes were observed for other cell lineages specific miRNAs, such as miR-146 for T lymphocyte, miR-107 for monocytes, miR-203 for epidermal cell, miR-206 for myogenic cells, miR-124 for neural cells and miR-126 for endothelial cells (Table 3). Taken together, these findings confirm the specificity of SMC differentiation. More important, the data suggested that miR-34a, a reported cell cycle mediator (He et al., 2007), was up-regulated to a greater magnitude over 8 days of differentiation than some best known SMC differentiation related miRNAs miR-143 and miR-145. Expression level of miR-34a during SMC differentiation was further validated using real-time PCR analysis, and the result showed that miR-34a expression was significantly increased during SMC differentiation (Figure 10), indicating that miR-34a may be involved in the SMC differentiation system.

Name	Fluorescence signal		Reported functions	Reference
	Day4/day0	Day8/day0		
miR-290-5P	0.775761	0.286816	ES cell specific	Houbaviy et al(2003)
miR-291a-5P	0.925415	0.271932	ES cell specific	Houbaviy et al(2003)
miR-292-3P	1.427017	0.468515	ES cell specific	Houbaviy et al(2003)
miR-293	1.18702	0.411204	ES cell specific	Houbaviy et al(2003)
miR-294	1.146818	1.367687	ES cell specific	Houbaviy et al(2003)
miR-295	1.062308	0.265621	ES cell specific	Houbaviy et al(2003)
miR-143	1.470071	1.237568	SMC differentiation	Cordes et al(2009)
miR-145	2.113956	1.460188	SMC differentiation	Cordes et al(2009)
miR-21	UD	9.848351	SMC proliferation	Ji et al(2007)
miR-22	2.20227	11.205712	Tumour suppressor	Xiong et al(2010)
miR-24	0.987122	3.597099	Heart contraction and myoblast differentiation	Li et al (2013b), Sun et al(2008)
miR-214	3.451106	2.024559	Muscle cell specification and myogenesis	Flynt et al(2007), Liu et al(2010)
miR-146a	1.225387	UD	T lymphopoiesis	Georgantas et al(2007)
miR-16	0.668986	1.187912	Universal expression	Landgraf et al (2007)
miR-107	0.754878	0.861935	Monocytopoiesis	Georgantas et al(2007)
miR-203	1.103917	UD	Epidermal differentiation	Yi et al(2008)
miR-206	1.563997	1.063436	Myogenic cells	Rao et al(2006), Kim et al(2006)
miR-124	0.707948	0.458512	Brain specific	Landgraf et al (2007)
miR-126-3P	1.455875	UD	Endothelial cells	Fish et al(2008)

miR-133a	2.509492	1.42217	Cardiac cells	Ivey et al(2008)
miR-133b	2.094272	1.293552	Cardiac cells	Ivey et al(2008)
miR-34a	3.763473	4.818876	cell cycle arrest	He et al(2007), Yamakuchi et al(2008), Chang et al(2007). Raver-Shapria et al(2007)
miR-34b-5P	1.869908	UD	cell cycle arrest	He et al(2007)
miR-34c-5P	1.726008	1.060224	cell cycle arrest	He et al(2007)

Table 3 Selected miRNAs expression profile during SMC differentiation from ES cells

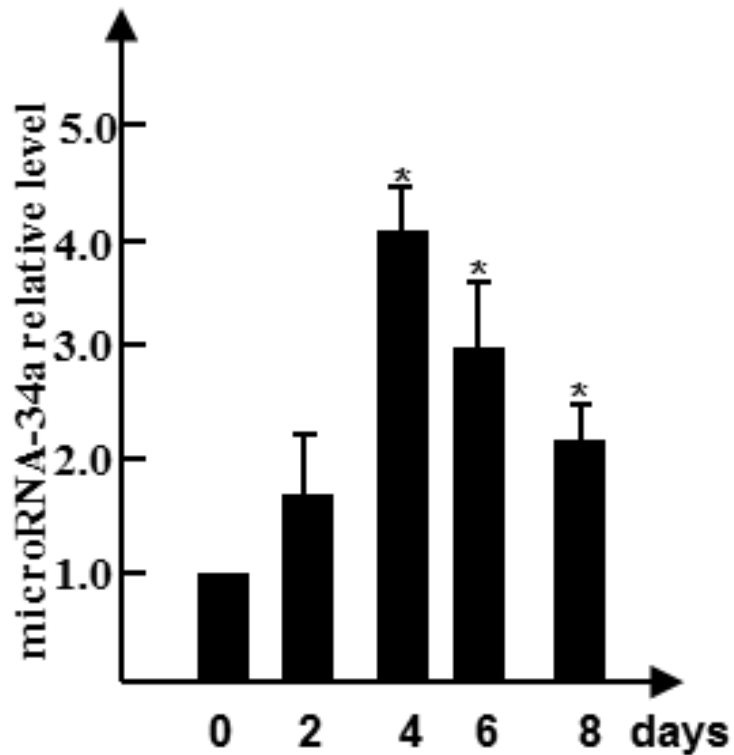


Figure 10 Real-Time PCR analysis of miR-34a during SMC differentiation

Undifferentiated ES cells were seeded on collagen coated flasks and cultured for 2, 4, 6 and 8 days in differentiation medium. Day 0 represented undifferentiated ES cells. Cells were harvested at indicated time points to extracted total RNA including small RNA, and subjected to real-time PCR analysis. The data represent mean \pm SEM of three independent experiments. * $P < 0.05$ (verse day 0)

4.1.2 Investigating the role of miR-34a in SMC differentiation *in vitro* by miRNA gain/loss-of function analyses

To investigate whether miR-34a promotes SMC differentiation, gain-of-function experiments were carried out by using Pre-miR™ mmu-miR-34a miRNA Precursor (Ambion) in differentiating ES cells as described in Chapter 2. Real-time PCR result showed that miR-34a expression was significantly increased after transfection, and miR-34a overexpression significantly up-regulated the expression of SMC-specific genes, such as SM α A, SH22 α , h1-calponin and SM-myh11 (Figure 11A). Western blot analysis was also carried out to determine whether protein level of SMC markers was affected by overexpression of miR-34a during SMC differentiation. Data from Figure 11B showed that the protein levels of SM α A and SM-MHC were significantly up-regulated in miR-34a overexpression groups, as compared to negative control group

To further test whether activation of miR-34a is essential for SMC differentiation, lose-of-function experiments were performed by using Anti-miR™ miR-34a inhibitor (Ambion) in differentiating ES cells. As miR-145 is a well-known SMC differentiation related miRNA, MISSION® microRNA Inhibitor has-miR-145-5p (Sigma-Aldrich) was used as positive control. The study showed that knockdown of miR-34a during differentiation inhibited the expression of SMC specific markers at

both RNA (Figure 12A) and protein levels (Figure 12B). Taken together, these data suggest that miR-34a plays an important role in SMC differentiation from ES cells.

To further understand the molecular mechanism by which miR-34a regulated SMC differentiation from ES cells, differentiating ES cells were transfected with miR-34a precursor or negative control, and subjected to microarray analyses performed by GENERGY BIO. (Beadchip: mouseWG-6_V2_0_R311278593-A;) to examine the gene expression profiling that affected by miR-34a overexpression during SMC differentiation. Data (Listed in appendix) demonstrated that a panel of SMC specific genes such as CNN1, Acta2, Tagln2, Aebp1, Smtn, Des, Cald1, Mylk Myh11 and Vcl (highlighted) were positively regulated by miR-34a overexpression. More importantly, several SMC transcription factors including SRF, Myocardin and MEF2c were also up-regulated by miR-34a, alongside SirT1. Therefore, Real-Time PCR and western blot analysis on differentiating SMCs with miR-34a overexpression or inhibition were performed to confirm the findings from microarray. The result suggested that overexpression of miR-34a up-regulated SRF, myocardin and MEF2C, while knockdown of miR-34a down-regulated these genes (Figure 13A and Figure 14A). Similar results were obtained from protein analysis, while protein levels of SRF and MEF2c were up-regulated by miR-34a overexpression but down-regulated by knockdown of miR-34a (Figure 13B and Figure 14B).

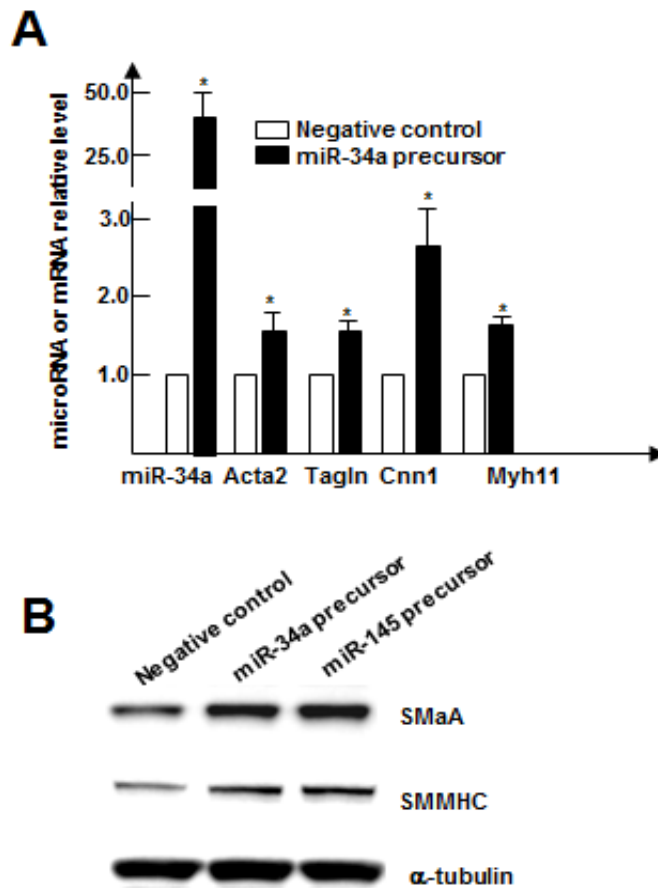


Figure 11 miR-34a overexpression up-regulates the expression of SMC markers in SMC differentiation

Undifferentiated ES cells were seeded on collagen coated flasks and cultured in differentiation medium. Day 2~3 differentiating ES cells were transfected with miR-34a precursor or negative control and cultured for another 48 to 72 hours. Cells were harvested and subjected to real-time PCR and western blot analysis, respectively. (A) Real-time PCR analysis of SMC gene expression after miR-34a overexpression. (B) Western blot analysis of SMC markers after miR-34a overexpression. miR-145 precursor was included as positive control. α -tubulin was

used as housekeeping protein. The data presented here are representative of mean \pm SEM of three to six independent experiments. *P<0.05 (versus control)

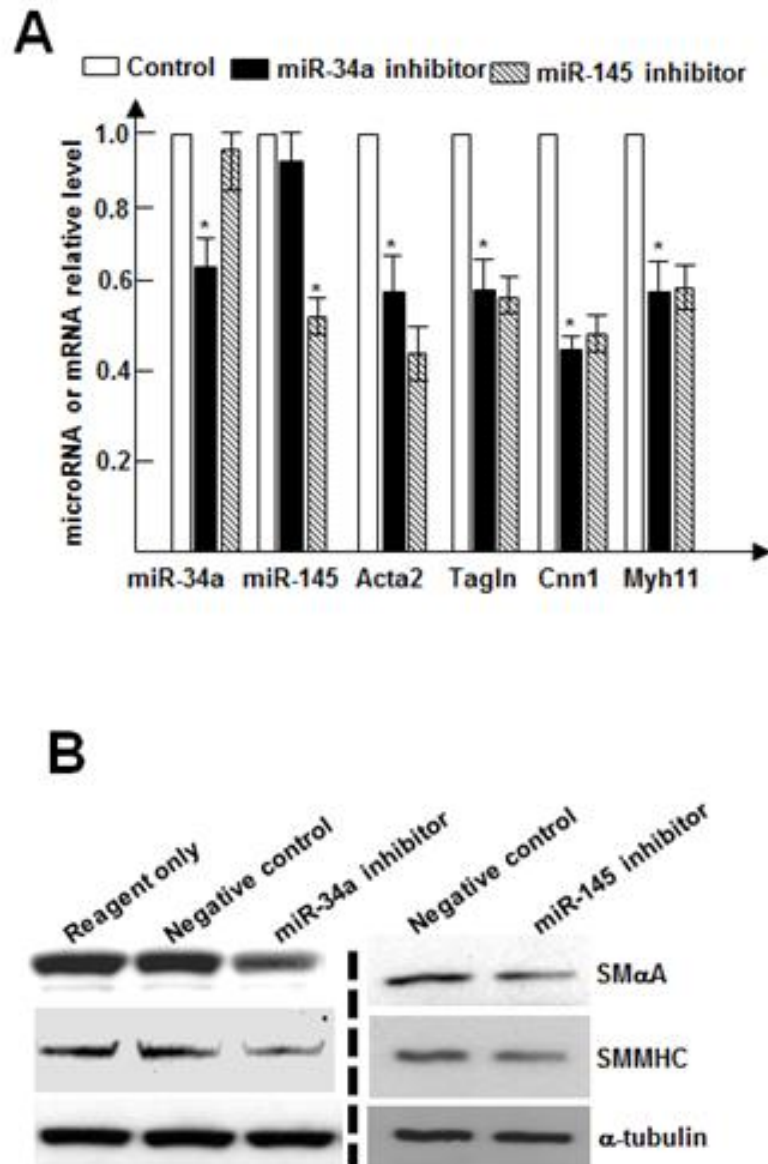


Figure 12 miR-34a knockdown inhibit expression of SMC markers in SMC differentiation

Day 2~3 differentiating ES cells were transfected with miR-34a inhibitor or negative control. miR-145 inhibitor was included as positive control. Differentiating ES cells were seeded on collagen coated flasks and cultured in differentiation medium for 48 hours after transfection. Cells were harvested and subjected to real-time PCR and

western blot analysis, respectively. (A) Real-Time PCR analysis of SMC gene and miRNA expression level in miR-34a knockdown experiment. (B) Western blot analysis of SMC markers protein expression with inhibition of miR-34a, where α -tubulin was used as housekeeping protein. The data presented here are representative or mean \pm SEM of three to six independent experiments. * $P < 0.05$ (versus control)

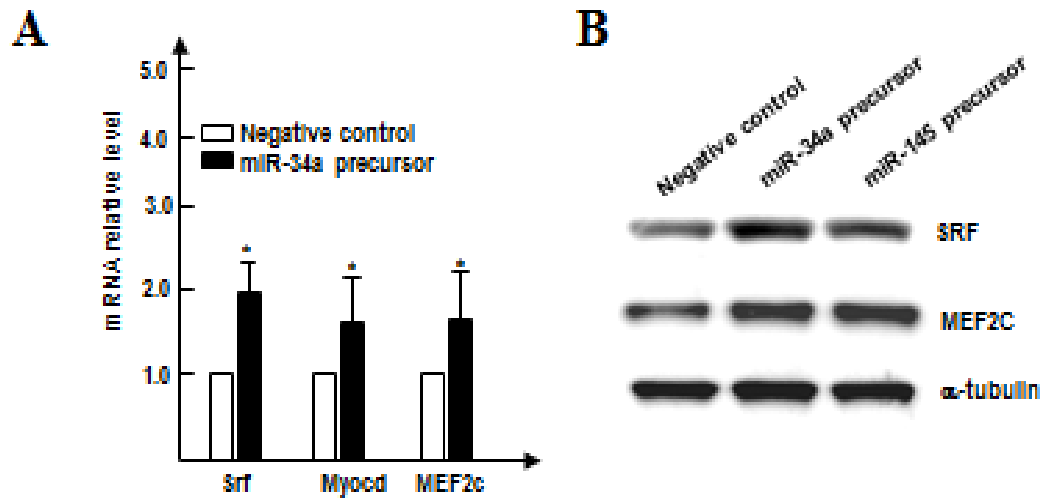


Figure 13 Overexpression of miR-34a positively regulated SMC specific transcription factors

Day 2~3 differentiating ES cells were transfected with miR-34a precursor or negative control and cultured for further 48 hours. Cells were harvested and subjected to real-time PCR and western blot analysis, respectively. (A) Real-Time PCR analysis of SMC transcription factor gene expression, which was up-regulated in miR-34a overexpression experiments. (B). Western blot analysis of SRF and MEF2c protein level. Protein level of SRF and MEF2c was up-regulated by overexpression of miR-34a. miR-145 precursor treatment was included as positive control. α -tubulin was used as housekeeping protein. The data presented here are representative or mean \pm SEM of three independent experiments. *P<0.05 (versus control)

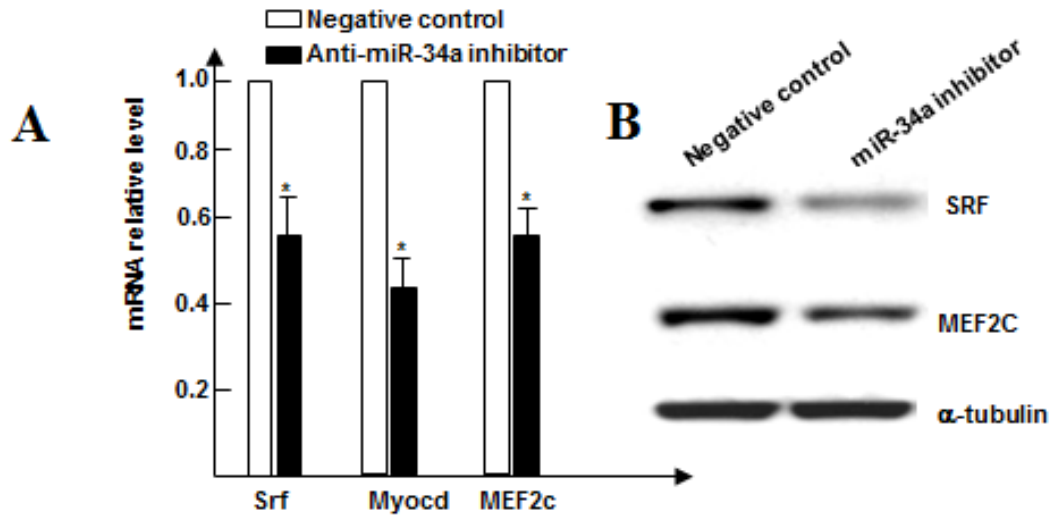


Figure 14 Knockdown of miR-34a down-regulated the expression levels of SMC transcription factors

Day 2~3 differentiating ES cells were transfected with miR-34a inhibitor or negative control. Differentiating ES cells were cultured in collagen coated flasks in differentiation medium for 48 hours after transfection. Cells were harvested and subjected to real-time PCR and western blot analysis, respectively. (A) Real-Time PCR analysis of SMC transcription factor gene expression from miR-34a knockdown experiments. (B) Western blot analysis of SMC transcription factor protein level from miR-34a knockdown experiments. The data presented here are representative or mean \pm SEM of three independent experiments. *P<0.05 (versus control)

4.1.3 miR-34a is involved in SMC differentiation *in vivo*

To carry out *in vivo* cell implantation study, miR-34a overexpressing (pLL3.7 – GFP-miR-34a) and control (pLL3.7-GFP) ES cell lines were generated by using miR-34a overexpression (pLL3.7–GFP-miR-34a) and control (pLL3.7-GFP) lentivirus respectively, as described in chapter 2. GFP-positive cells with high purity were sorted out (Figure 15A) and maintained in ES cell culture medium. No significant differences were observed between sorted cells and their parent undifferentiated ES cells in terms of morphology, self-renew and pluripotency when they were maintained in ES cell culture medium for up to 5 passages. The miR-34a overexpressing and control ES cells were induced to differentiate SMCs using *in vitro* SMC differentiation method as described above. Cells were harvested at day 0 and 8 post-differentiation and subjected to real-time PCR and flow cytometry analysis, respectively. Compared to control ES cells, the expression of miR-34a in miR-34a overexpression ES cells was slightly higher at day 0 (undifferentiated cells), but significantly increased at day 8 of differentiation (Figure 15B), suggesting that miR-34a expression was successfully up-regulated in these cells during SMC differentiation. Importantly, more SMCs were differentiated from miR-34a overexpression ES cells as compared with control ES cells at day 8 (Figure 15C), further confirming the important role of miR-34a in SMC differentiation.

To determine the functional importance of miR-34a in SMC differentiation *in vivo*, pLL-3.7-GFP-miR-34a and pLL-3.7-GFP ES cells were subcutaneously injected into C57BL/6J mice with 100ng/ml of PDGF-BB to promote *in vivo* SMC differentiation as described in chapter 2. As expected, Results from Immunofluorescence staining showed that the majority of cells within Matrigel implants from both treatments were GFP-positive (Green signal), indicating its exogenous origins. Importantly, more SM-MHC positive cells were observed in the implants of miR-34a overexpressing ES cells (Figure 16A and B). Furthermore, the expression of miR-34a, SM α A, SM-MHC and SirT1 in Matrigel implants of miR-34a overexpression ES cells was significantly higher than that of control ES cells, as demonstrated in both real-time PCR (Figure 17A) western blot analysis (Figure 17B). Taken together, these data suggest an important role of miR-34a in SMC differentiation from ES cells *in vivo*.

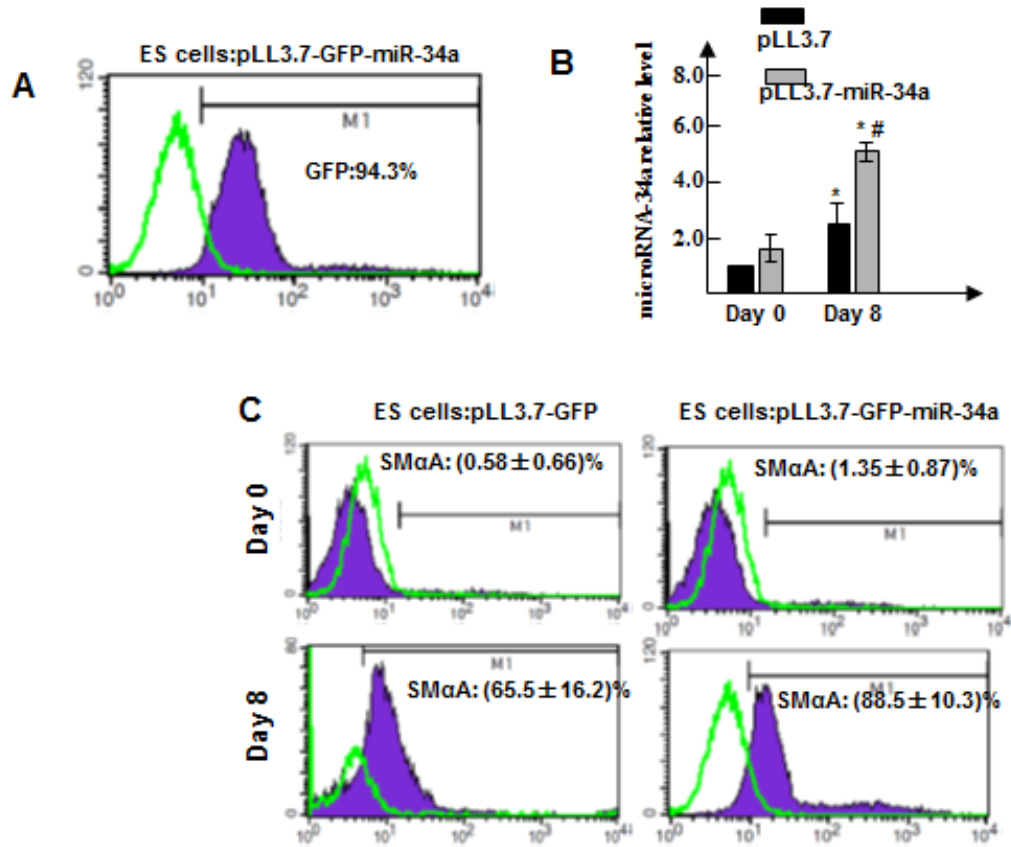


Figure 15 Generation of miR-34a over expression ES cells and differentiation towards SMCs

(A) miR-34a overexpression and control ES cell line were generated as described in chapter 2. Higher than 94% of sorted cells were GFP-positive as examined by flow cytometry analyses . (B) ES cell lines were cultured in collagen coated flasks in differentiation medium for 8 days. Cells were harvest and subjected to real-time PCR analysis. *P<0.05 (versus day 0), #P<0.05 (pLL3.7-GFP-miR-34a versus pLL3.7 GFP). (C) After 8 days of differentiation, more SMCs were differentiated from miR-34a overexpressing ES cells. Data presented here are representative or mean \pm SEM of three independent experiments.

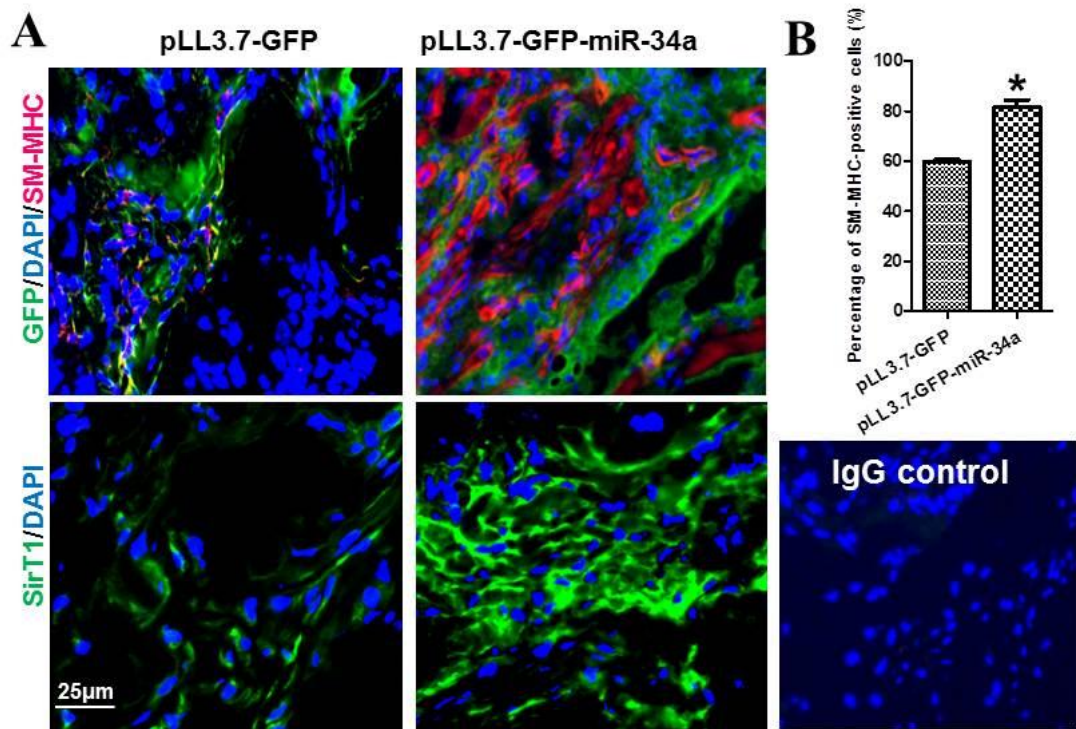


Figure 16 miR-34a promotes SMC differentiation from ES cells *in vivo*

Matrigel plugs implanted with miR-34a overexpressing (pLL-GFP-miR-34a) or control (pLL-3.7-GFP) ES cells were harvested, sectioned and subjected to immunofluorescence staining using antibodies against GFP and SM-MHC or SirT1 alone. (A) Representative images from immunofluorescence staining, Green: GFP or SirT; Red: SM-MHC; Blue: DAPI. (B) Quantitative data of the percentage of SM-MHC positive cells. The percentage of GFP-labeled SM-MHC positive cells per field was counted by two trained independent investigators blinded to the treatment from four random high power fields (200x) in each sections, three sections from each implant and four implants from each group.*P<0.05

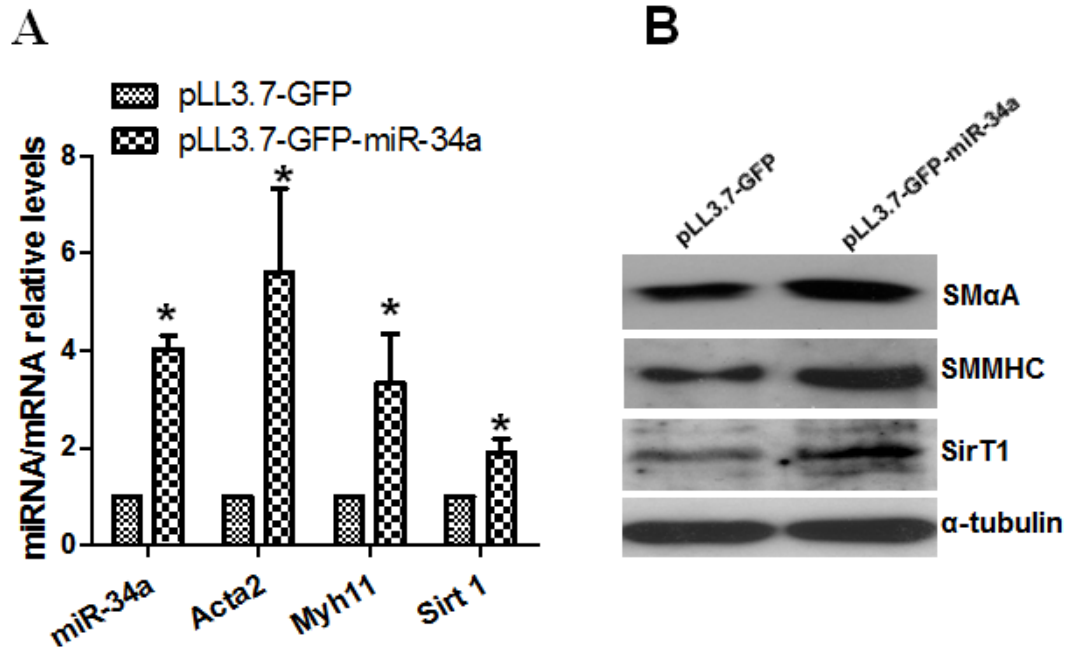


Figure 17. SMC differentiation was up-regulated by miR-34a overexpression *in vivo*

Matrigel plugs implanted with miR-34a overexpressing (pLL-GFP-miR-34a) or control (pLL-3.7-GFP) ES cells were harvested. Total RNA including small RNA, and protein were extracted from Matrigel implants and subjected to real-time PCR (A) and western blot (B) analysis, respectively. α -tubulin was used as housekeeping protein. The data presented here are representative or mean \pm of four Matrigel implants. *P<0.05

4.1.4 miR-34a also plays an important role in SMC differentiation from human ES cells.

As the current result suggested that miR-34a plays an important role in SMC differentiation from mouse ES cells, it was still not clear that whether miR-34a was involved in SMC differentiation from human ES cells. To address this issue, human ES cells were induced to differentiate towards SMC by using a similar differentiation method as used in the mouse ES cell differentiation study. The result demonstrated that human ES cells can be successfully differentiated into SMCs, based on the data from real-time PCR (Figure 18A), Western blot (Figure 18B), immunofluorescence staining (Figure 18C) and flow cytometry (Figure 18D) analyses. miR-34a level was also significantly increased during SMC differentiation from human ES cells (Figure 19A). Similar to the result obtained from murine ES cells, SMC markers was up-regulated by miR-34a overexpression (Figure 19B and C), while miR-34a knockdown inhibited SMC marker expression in differentiating human ES cells (Figure 19D and E). These finding further confirmed a critical role of miR-34a in human SMC differentiation.

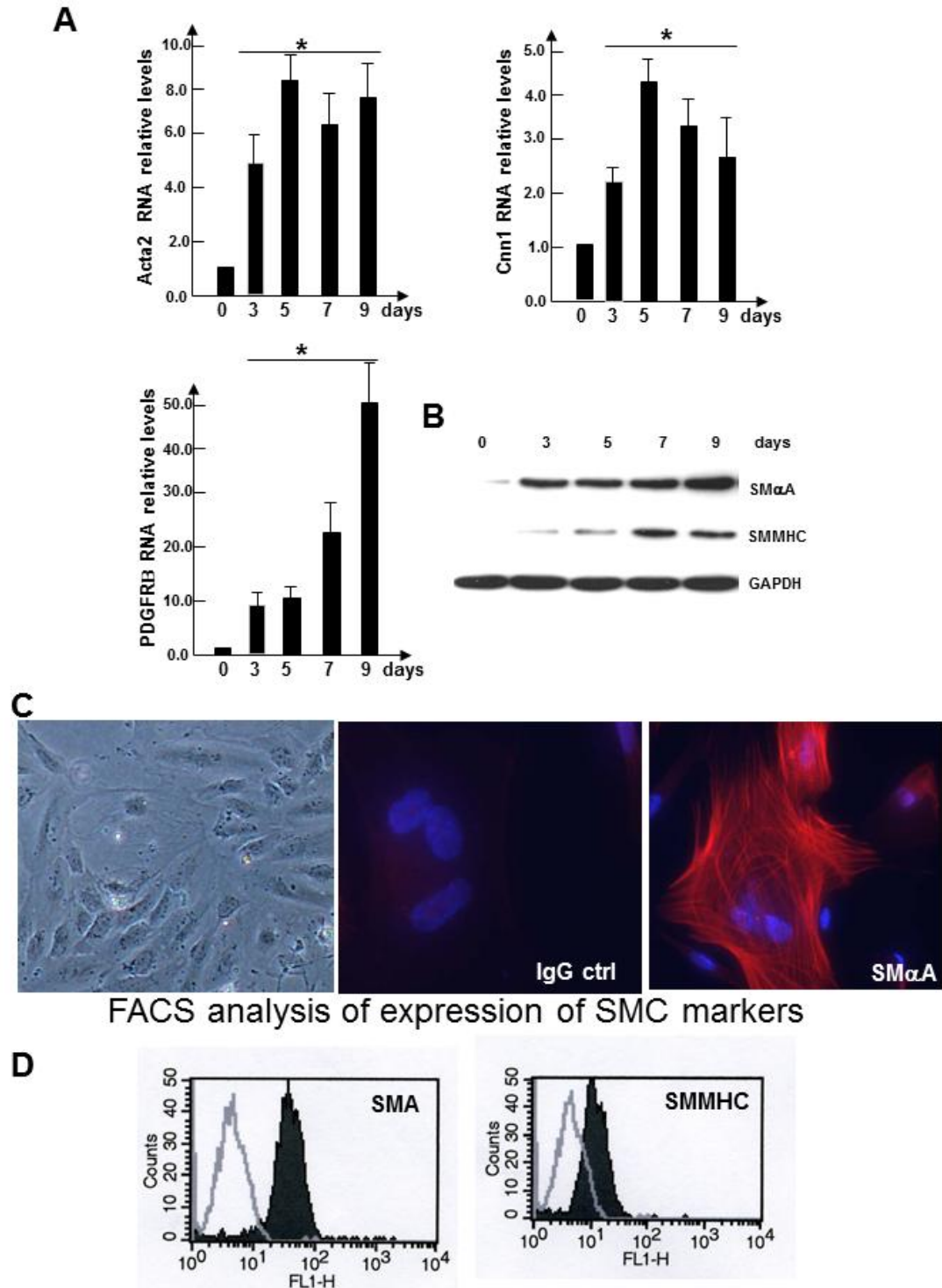


Figure 18 SMC differentiation from human ES cells *in vitro*

Undifferentiated human ES cells were seeded into collagen coated flasks and cultured in differentiation medium for 3, 5, 7 and 9 days respectively. Total RNA

(including small RNA) and protein were extracted and subjected to real-time PCR (A) and Western blot (B) analysis, respectively. Day 0 represented undifferentiated human ES cells. (C) Immunofluorescence analysis of SM α A expression in differentiated SMCs (day 9). Representative of phase-control image and IgG control were also included. (D) Flow cytometry analyses of SMC markers expression in differentiated SMCs from human ES cells by using antibodies against SM α A or SM-MHC. The data presented here are representative of mean \pm SEM of three independent experiments. *P<0.05 (versus day 0)

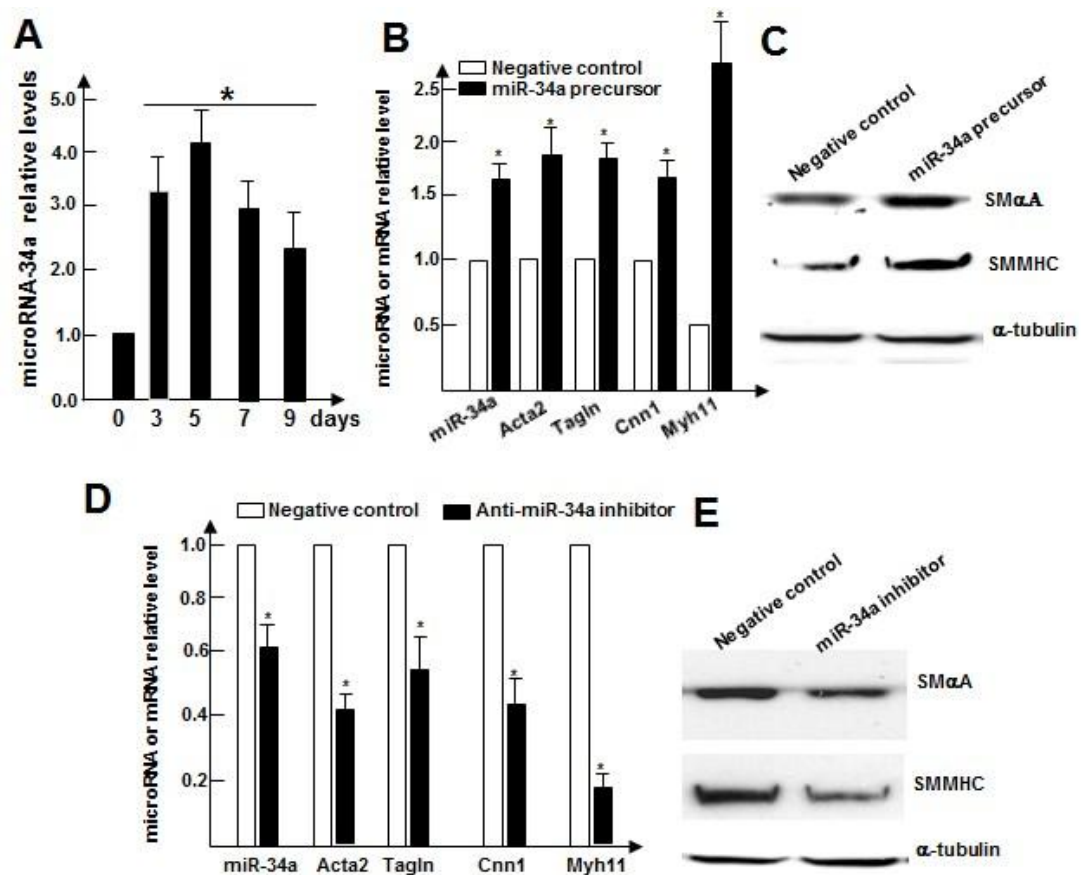


Figure 19 SMC differentiation from human ES cells can be regulated by miR-34a

(A) Expression of miR-34a was up-regulated during SMC differentiation from human ES cells. Undifferentiated human ES cells were seeded into collagen coated flasks and cultured in differentiation medium for 3, 5, 7 and 9 days, respectively. Total RNA (including small RNA) was extracted and subjected to real-time PCR analysis. * $P < 0.05$ (verse day 0). (B-E) Day 2 ~3 differentiating human ES cells were transfected with miR-34a precursor or inhibitor, or respective negative controls then cultured in SMC differentiation medium for 48 to 72 hours after transfection. Total RNA (including small RNA) and protein were extracted and subjected to Real-Time

PCR (B and D) and Western blot (C and E) analysis, respectively. Expression of SMC specific marker was up-regulated by miR-34a overexpression (B and C), and inhibited by knockdown of miR-34a (D and E).The data presented here are representative or mean \pm of three independent experiments (n=3). *P<0.05 (versus negative control).

4.2 SirT1 is identified as the mRNA target for miR-34a in SMC differentiation from ES cells

To further investigate the mechanism by which miR-34a regulates SMC differentiation from ES cells, potential mRNA targets of miR-34a were scrutinized and SirT1 was predicted as one of the top targets of miR-34a in several computational algorithmic databases, such as Targetscan (www.targetscan.org), pictar (www.pictar.mdc-berlin.de), and miRanda (www.microrna.org). Two highly conserved binding sites for 5' end (code sequence) of miR-34a were located on the 3'UTR of SirT1 (Figure 21A)

4.2.1 SirT1 was selected as potential mRNA target for miR-34a

To investigate whether SirT1 is the mRNA target for miR-34a, SirT1 gene expression was analyzed during SMC differentiation from ES cells. Unexpectedly, SirT1 gene expression was up-regulated (Figure 20A) and displayed a positive correlation with expression of miR-34a during SMC differentiation (Figure 10), suggesting that miR-34a may positively regulated SirT1 gene expression. This hypothesis was supported by the findings that SirT1 gene and protein levels were significantly up-regulated by miR-34a overexpression, while inhibition of miR-34a down-regulated SirT1 expression levels (Figure 20B and C). These data suggested

that SirT1 is positively regulated by SirT1 directly or indirectly.

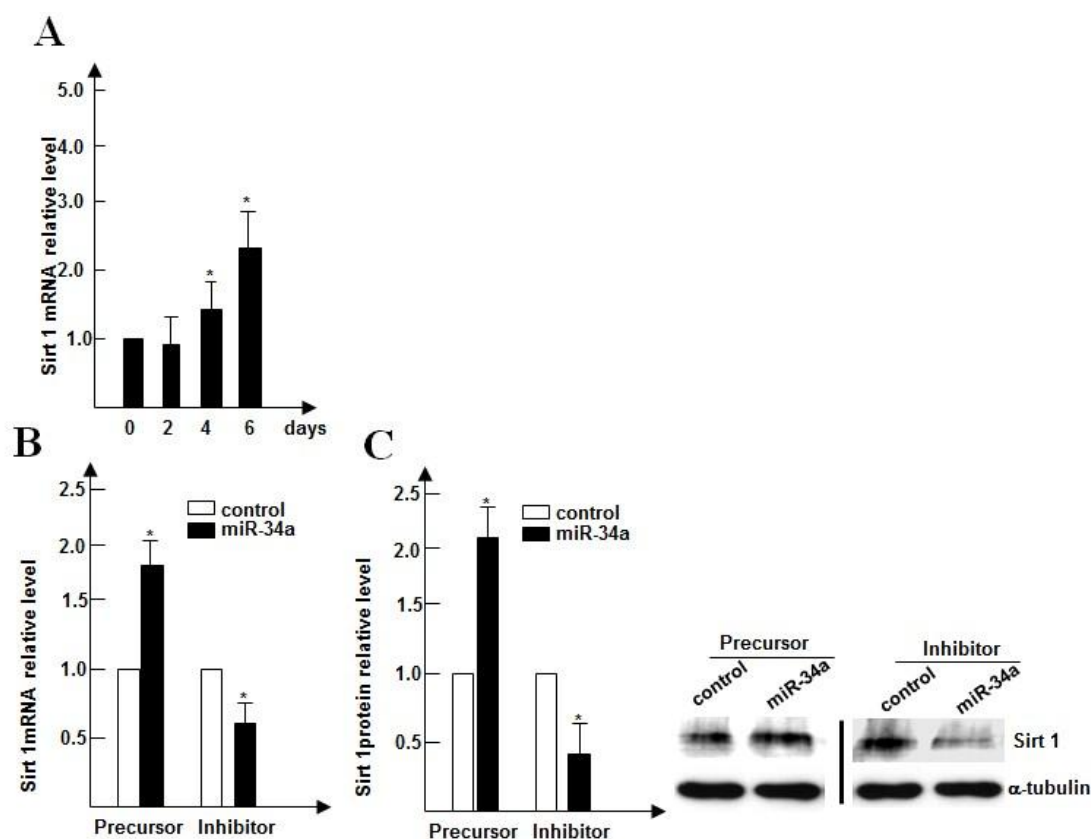


Figure 20 SirT1 is the potential mRNA target for miR-34a

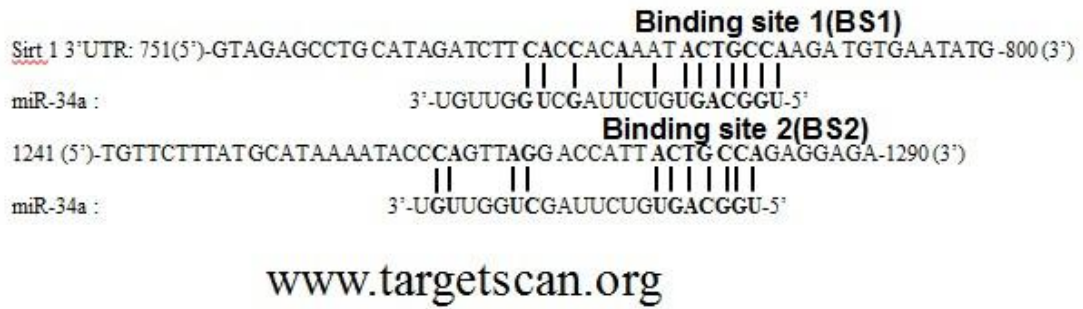
(A) Undifferentiated ES cells were seeded on collagen coated flasks and cultured in differentiation medium for 2, 4 and 6 days. Day 0 represented undifferentiated ES cells. Total RNA was extracted and subjected to Real-Time PCR analyses. SirT1 expression was up-regulated during SMC differentiation. (B-C) SirT1 is positively regulated by miR-34a. Day 2~ 3 differentiating ES cells were transfected with miR-34a precursor or miR-34a inhibitor with respective negative control then cultured in differentiation medium for 48 hours. Total RNA (including small RNA)

and protein were extracted and subjected to Real-Time PCR (B) and Western blot (C) analyses, respectively. The data presented here are representative or mean \pm S.E.M. of three to four independent experiments. *P<0.05 (versus day 0) in (A). *P<0.05 (treatment verse control) in (B and C)

4.2.2 Identifying SirT1 as mRNA target for miR-34a

To further confirm whether SirT1 is the mRNA target for miR-34a during SMC differentiation, the 3'UTR of SirT1 which contained two binding sites for miR-34a was cloned into a luciferase reporter (pmiR-Luc-SirT1-WT). This reporter was co-transfected with miR-34a precursor or negative control into differentiating ES cells. Result from miRNA reporter assay showed that the activity of luciferase from construct harboring the 3'UTR was significantly up-regulated by overexpression of miR-34a. To better understand this result, mutation of the binding sites for miR-34a on the 3'UTR of SirT1 from pmiR-Luc-SirT1-WT was carried out to generate luciferase reporter containing individual binding site mutation (pmiR-Luc-SirT1-BS1^{mu} and pmiR-Luc-SirT1-BS2^{mu}), or dual binding sites mutation (pmiR-Luc-SirT1-BS1/2^{mu}) by site-direct mutagenesis. These miRNA reporters were transfected into differentiating ES cells with miR-34a precursor, and the result showed that luciferase activities of reporter containing binding site 2 mutation(BS2^{mu}) or dual binding sites mutation(BS1/2^{mu}) were not up-regulated by miR-34a overexpression, indicating that the binding site 2 is mainly responsible for the up-regulation of SirT1 3'UTR reporter luciferase activity which mediated by miR-34a overexpression. Taken together, these data has firmly confirmed that SirT1 is the mRNA target, which is positively regulated by miR-34a during SMC differentiation.

A



B

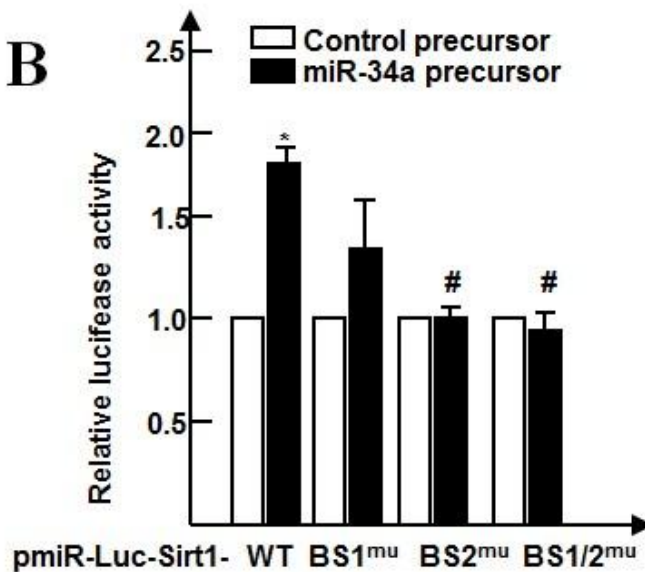


Figure 21 SirT1 is identified as the mRNA target for miR-34a during SMC differentiation

(A) Two potential binding sites for miR-34a on 3'UTR of SirT1 predicted by *Targetscan* are depicted in this illustration. (B) miR-34a binding site 2 on 3'UTR of SirT1 is required for miR-34a mediated SirT1 gene activation. Wild type SirT1 3'UTR reporter (pmir-Luc-SirT1-WT) or three mutants (pmir-Luc-SirT1-BS1^{mu}, pmir-Luc-SirT1-BS2^{mu} and pmir-Luc-SirT1-BS1/2^{mu}) were co-transfected with miR-34a precursor or negative control into day 2~3 differentiating ES cells and

luciferase activity was measured 48 hours after transfection. Reporter luciferase activities were up-regulated by miR-34a overexpression in WT and BS1^{mu} group, but not in BS2^{mu} or BS1/2^{mu} group. The data presented here are mean \pm S.E.M. of four independent experiments. *P<0.05 (treatment versus control) # P<0.05 (binding site mutants versus wild type).

4.3 miR-34a regulates SirT1 and cell cycle during SMC differentiation

Current data suggested that SirT1 is the mRNA target of miR-34a and can be up-regulated by miR-34a during SMC differentiation. However, the mechanism by which miR-34a positively regulates SirT1 is still not clear and requires more investigation.

4.3.1 miR-34a differently regulates SirT1 gene expression in undifferentiated ES cells and differentiated SMCs

In order to investigate if miR-34a up-regulated the expression of its target gene, SirT1, during SMC differentiation is cellular context specific, miR-34a over-expression or inhibition experiments and luciferase activity assays were conducted in naïve stem cells (undifferentiated ES cells) and terminally differentiated SMCs {It has been reported that day 8 differentiated SMCs are fully/terminally differentiated SMCs(Xiao et al., 2009)}, respectively. Interestingly, the data showed that SirT1 3'UTR reporter activity was significantly suppressed by miR-34a overexpression, but up-regulated by inhibition of miR-34a in undifferentiated ES cells (Figure 22A). As expected, SirT1 3'UTR reporter activity was significantly up-regulated by miR-34a overexpression, and decreased by knockdown of miR-34a in terminally differentiated SMCs (Figure 22B). These findings suggested that miR-34a regulates SirT1 gene expression is cellular context dependent.

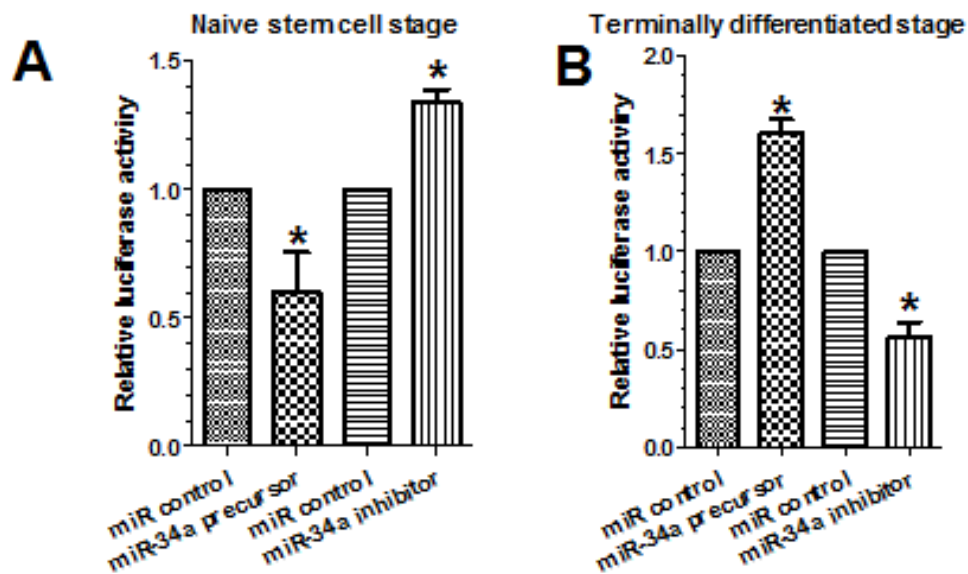


Figure 22 SirT1 3'UTR reporter activity in naive stem cell or terminally differentiate SMCs.

(A) Undifferentiated ES cells were transfected with pmiR-Luc-SirT1-WT as well as miR-34a precursor, miR-34a inhibitor or respective negative control. Luciferase activity was measured 48 hours after transfection. SirT1 3'UTR reporter activity was suppressed by miR-34a overexpression but increased by inhibition of miR-34a in naive stem cell. (B) Day 8 differentiated SMCs were transfected with pmiR-Luc-SirT1-WT as well as miR-34a precursor, miR-34a inhibitor or respective negative control. Luciferase activity was measured 48 hours after transfection. SirT-1 3'UTR reporter activity was up-regulated by miR-34a overexpression while knockdown of miR-34a decrease the reporter activity. The data presented here are mean \pm S.E.M. of four independent experiments. *P<0.05 (versus respective controls).

4.3.2 miR-34a binding site alone is not sufficient for miR-34a mediated SirT1 gene expression.

As the result showed that miR-34a binding sites on 3'UTR of SirT1 are required for miR-34a mediated SirT1 gene expression (Figure 21), the next step is to determine whether miR-34a binding site alone is sufficient for this regulation. A luciferase reporter was generated with an artificial binding site for miR-34a, but without surrounding RNA sequence and structure of SirT1 3'UTR, designated as pmiR-Luc-miR-34a binding site. The reporter was transfected into differentiating ES cells with miR-34a precursor or negative control. Data from Figure 23 showed that miR-34a can only slightly up-regulated the reporter activity but not reached significance. This result suggests that the surrounding RNA sequence and/or structure of SirT1 3'UTR are also required for miR-34a mediated SirT1 up-regulation.

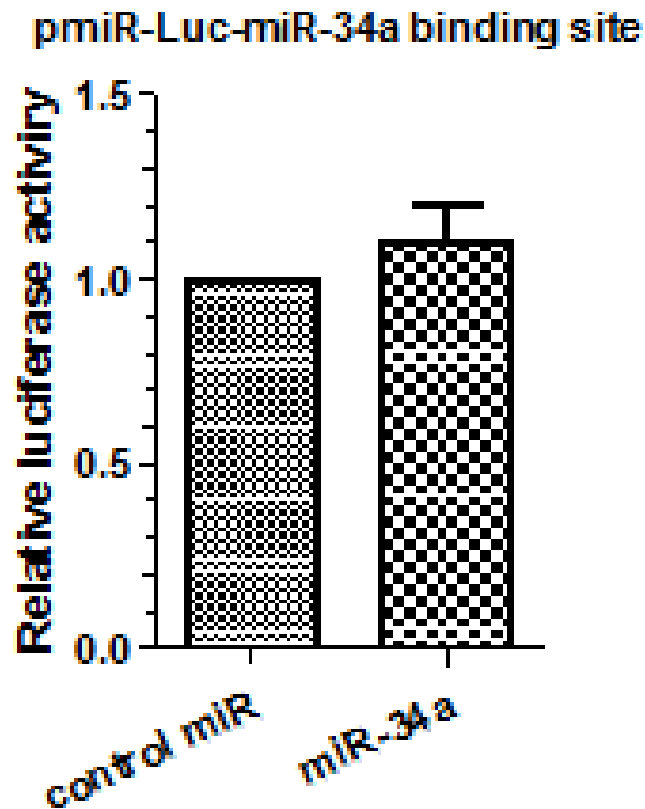


Figure 23 miR-34a binding site alone is not sufficient for SirT1 up-regulation

pmiR-Luc-miR-34a binding site was generated as described in chapter 2 and transfected into day 2 differentiating ES cells in 24-well plate with miR-34a precursor or negative control. Luciferase assay was performed 48 hours after transfection. No significant difference on reporter activity was observed between control and miR-34a overexpression group. The data presented here are S.E.M. of three independent experiments.

4.3.3 miR-34a specifically up-regulates SirT1 during SMC differentiation

MECP2 has been suggested to play an important role in SMC differentiation from ES cells (personal communications), and it has been reported that ZEB1 is involved in endothelial cell differentiation from human ES cells (Luo et al., 2013). To investigate whether regulation of SirT1 by miR-34a is a specific but not globe effect during SMC differentiation, MECP2 (target gene for miR-22) and ZEB1 { target gene for miR-200C/150 (Luo et al., 2013)} luciferase reporters were transfected into differentiating ES cells combined with miR-34a precursor. Luciferase assay showed that there was no effects of miR-34a overexpression on neither reporter (Figure 24), further confirming that miR-34a specifically up-regulates SirT1 expression during SMC differentiation.

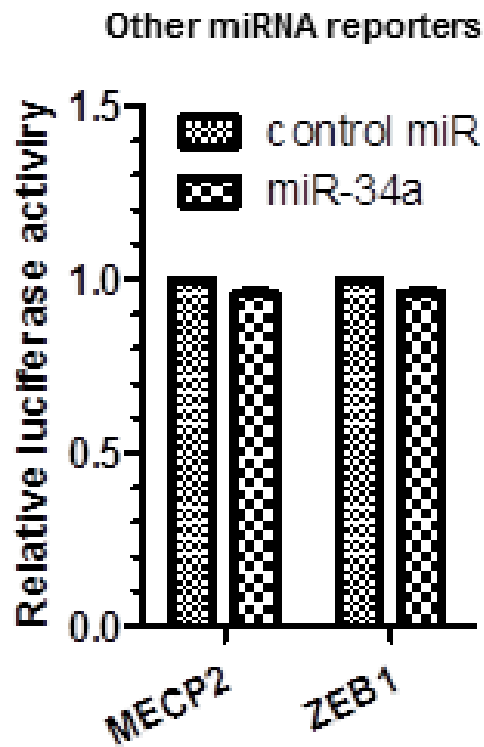


Figure 24 Other miRNA reporter were not affected by miR-34a over-expression

MECP2 reporter and ZEB1 reporter were transfected into day 2 differentiating ES cells with miR-34a precursor or negative control. Luciferase assay was performed 48 hours after transfection. No significant difference on luciferase activity between treatment and control. The data presented here are mean \pm S.E.M. of three independent experiments.

4.3.4 miR-34a promotes cell cycle arrest at G0/1 in differentiating ES cells

It has been reported that miRNA-target gene regulatory machinery will switch from translation repression to activation when cells are arrested at G0/1 phase, and miRNAs can regulate target gene translation activation in quiescent cells (Vasudevan et al., 2007, Vasudevan et al., 2008). Also, miR-34a has been reported to play an important role in regulating cell cycle in certain cell line (Sun et al., Cole et al., 2008, Chang et al., 2007). In this study less cell numbers were observed in miR-34a overexpression treatment during SMC differentiation (Figure 25). To investigate whether miR-34a is involved in cell cycle regulation during SMC differentiation, undifferentiated ES cells and differentiating SMCs were subjected to cell cycle analyses. The result showed that differentiating cells were gradually arrested at G0/1 phase during SMC differentiation (Figure 26). More importantly, this cell cycle arrest can be enhanced by miR-34a overexpression (Figure 27A), indicating that miR-34a may be involved in regulation of cell cycle during SMC differentiation. These findings were further confirmed by apoptosis analysis (Figure 27B) and BrdU incorporation assays (Figure 27C), which showed that miR-34a has no significant impact on cell apoptosis or death, and much less cells were entering S-phase upon miR-34a overexpression.

To further confirm whether up-regulation of SirT1 gene expression by miR-34a is specific to G0/1 cell cycle stage, differentiating ES cells were arrested at G0/1 phase by cell cycle arrest inducer aphidicolin and luciferase activity assays were performed. The result suggested that incubation of differentiating ES cells with 2 μ g/ml aphidicolin significantly increased cell cycle arrest at G0/G1 phase, and such arrest was further enhanced by miR-34a overexpression (Figure 28A). As a result, SirT1 3' UTR reporter activity was significantly increased by aphidicolin induced cycle cell arrest at G0/G1 phase and miR-34a overexpression in a similar manner (Figure 28B). Taken together, these data suggested that the miR-34a induced cell cycle arrest at G0/1 is, at least partially, responsible for the up-regulation of SirT1 during SMC differentiation.

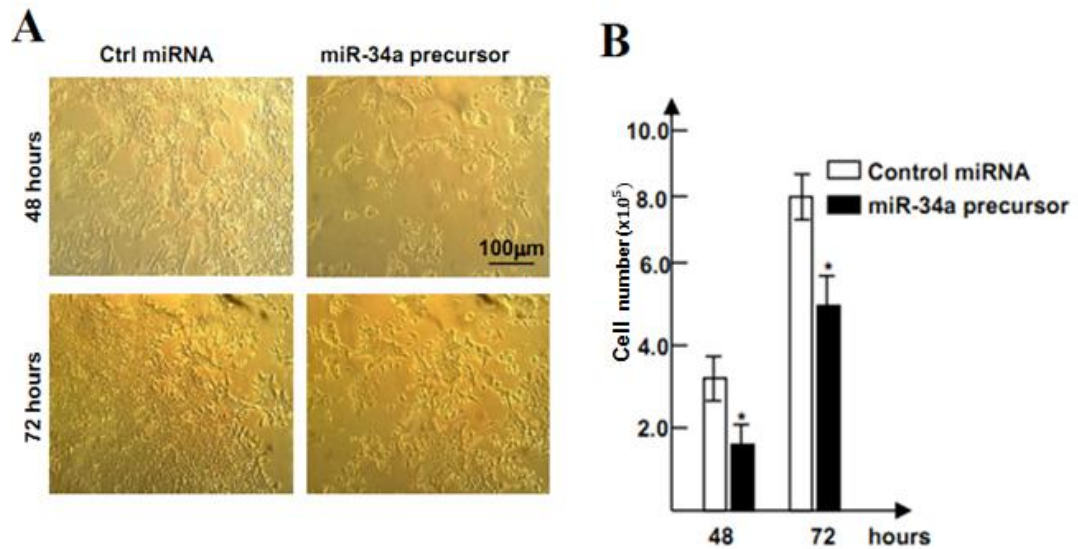


Figure 25 Less cell numbers were observed in the miR-34a over-expressing cells

Day 2~3 differentiating ES cells were transfected with miR-34a precursor or negative control and cultured in differentiation medium for 48 to 72 hours. (A) Representative microscopy images of differentiating ES cells with or without miR-34a overexpression. (B) Cells were dissociated into single cell suspension by trypsin-EDTA and counted by using a hemocytometer. The cells numbers were counted by two trained independent investigators blinded to the treatment, from 3 flasks for each treatment at indicated time point. *P<0.05 (versus control)

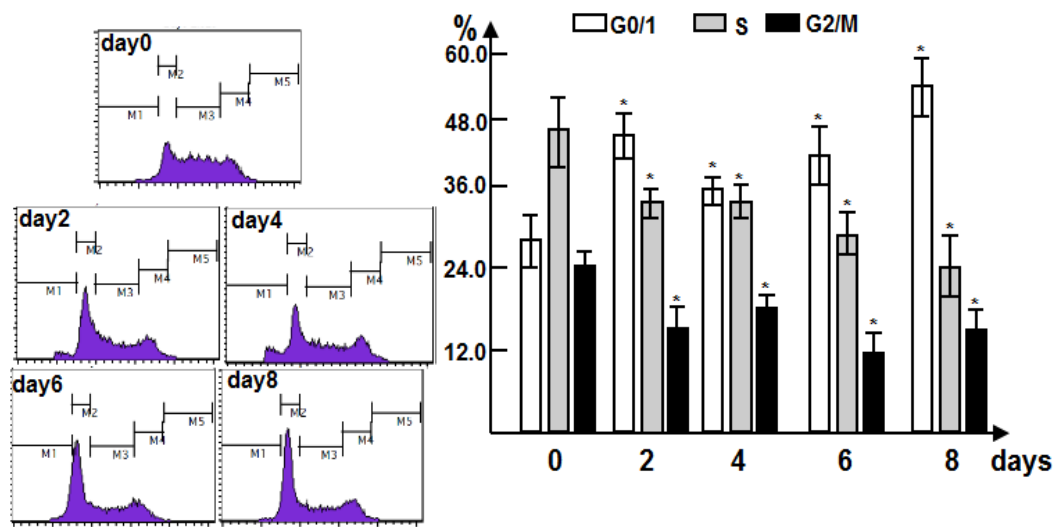


Figure 26 Cell cycle analysis during SMC differentiation

Undifferentiated (Day 0) ES cells were seeded on collagen coated flasks and cultured in differentiation medium for 2, 4, 6 and 8 days. Cells were harvested and subjected to cell cycle analysis. Representative of flow cytometry histogram and percentages of each cell cycle phase (G0/1, S and G2/M) were presented here. The data presented here are representative or mean \pm S.E.M. of three independent experiments *P<0.05 (versus day 0)

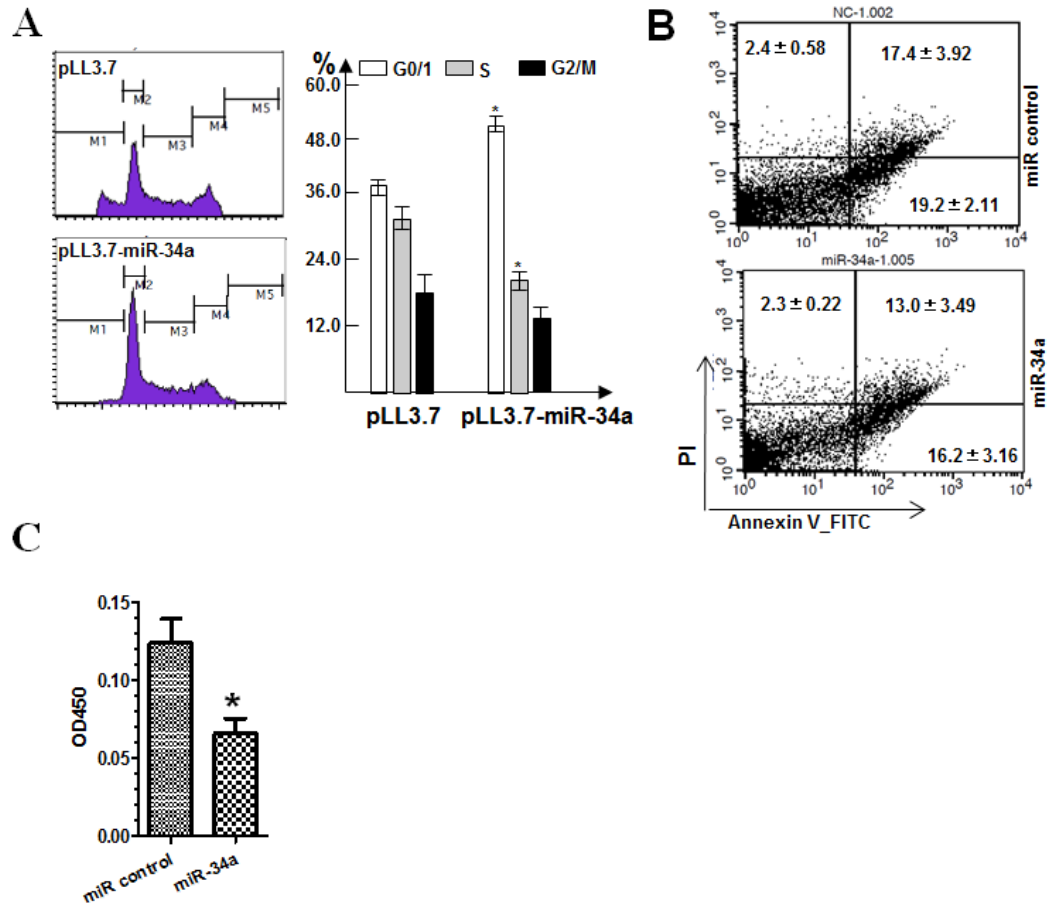


Figure 27 miR-34a promotes cell cycle arrest at G0/1 and inhibits cell proliferation

Day 2~3 differentiating ES cells were transfected with miR-34a precursor or negative control and cultured in differentiation medium for 48 hours. Cells were harvested and subjected to cell cycle (A), apoptosis (B) and BrdU incorporation (C) analyses, respectively. (A) miR-34a overexpression significantly promoted cell cycle arrest at G0/G1 phase. (B) miR-34a overexpression had no significant impact on cell apoptosis and death during SMC differentiation. (C) miR-34a overexpression inhibited differentiating ES cells into S-phase. The data presented here are

representative or mean \pm S.E.M. of three to four independent experiments.

* $P < 0.05$ (versus control)

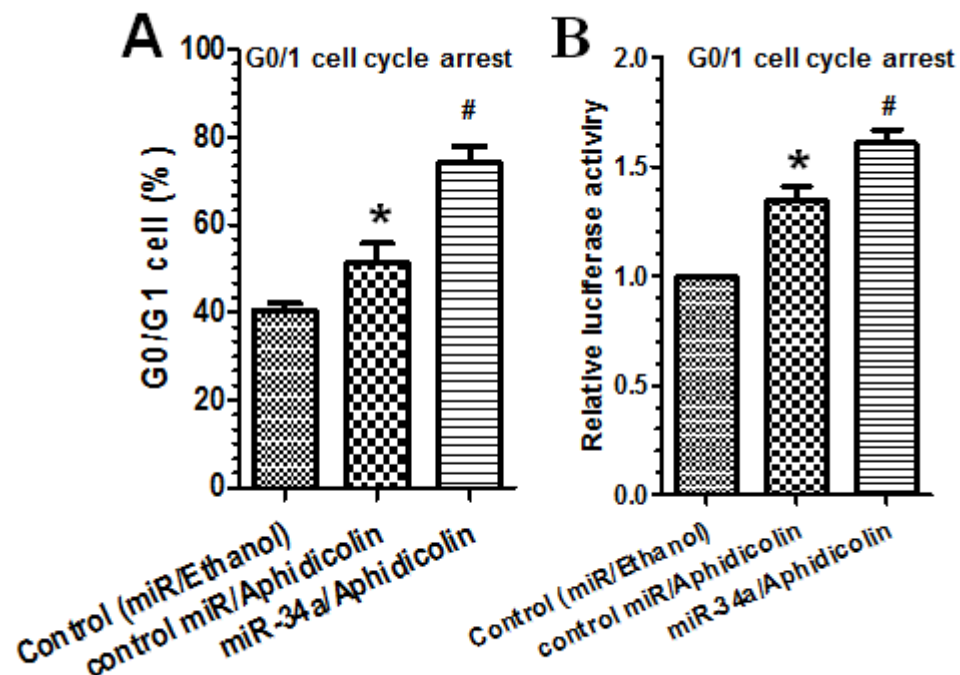


Figure 28 miR-34a mediated SirT1 gene activation was further enhanced by G0/G1 cell cycle arrest inducer

Sirt1 3'UTR wild type reporter was transfected into day 2 ~3 differentiating ES cells with miR-34a precursor or negative control and cultured for further 24 hours. 2 μ g/ml of aphidicolin or same amount of carrier vehicle (ethanol) was added into culture medium and incubated for 24 to 28 hours. Cells were harvested and subjected to cell cycle and luciferase assay. (A) Aphidicolin induced cell cycle arrest at G0/1 phase can be further enhanced by miR-34a overexpression. (B) miR-34a overexpression

further up-regulated SirT1 gene expression in the presence of G0/G1 inducer aphidicolin. The data presented here are mean \pm S.E.M. of three independent experiments. *P<0.05 (aphidicolin versus vehicle), #P<0.05 (miR-34a versus control).

4.3.5 Less miR-34a and SirT1 mRNA were incorporated into Ago 2-RISC complex upon differentiation

Ago 2 belongs to Argonaute proteins family. Ago 2 is one of the key components of RISC and have a critical role in miRNA induced gene silencing (Meister, 2013). To further investigate the molecular mechanism by which miR-34a up-regulates SirT1 gene expression during SMC differentiation, undifferentiated and differentiated SMCs were harvested and subjected to RNA-IP assays with antibody against Ago 2. The result showed that the enrichments of miR-34a and SirT1 3'UTR RNA (spanning around miR-34a binding sites 1 and 2) in undifferentiated ES cells were much higher than that of differentiated SMCs (Figure 29), indicating that less miR-34a and SirT1 were loaded into Ago 2 -RISC complex during SMC differentiation

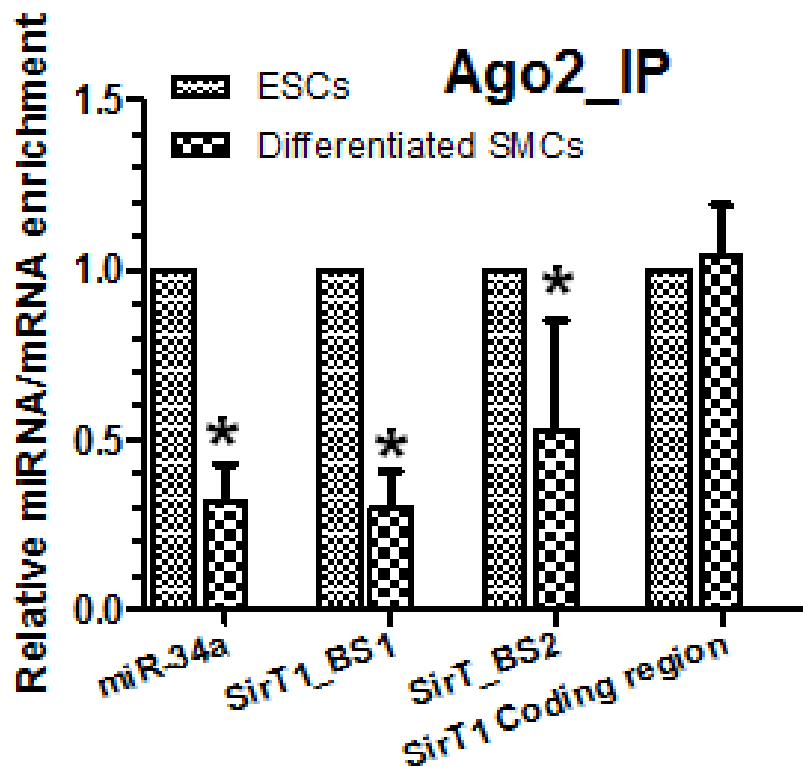


Figure 29 Less miR-34a and SirT1 were loaded onto Ago 2-RISC complex during SMC differentiation

Undifferentiated ES cells and differentiated SMCs were harvested and then subjected to RNA-IP assay with antibody against Ago 2 or normal rabbit IgG, respectively. Real-Time PCR amplification of SirT1 coding region was included as additional control for specific SirT1 3'UTR enrichment. The data presented here are mean \pm S.E.M. of three independent experiments. *P<0.05

4.4 Role of SirT1 in miR-34a induced SMC differentiation

As described above, SirT1 has been proved to be the mRNA target for miR-34a and up-regulated by miR-34a upon SMC differentiation. However, the role of SirT1 during SMC differentiation is still unclear, which requires further investigation in this section.

4.4.1 SirT1 activation is required for miR-34a mediated SMC differentiation

To investigate the potential role of SirT1 during SMC differentiation, SirT1 overexpression experiments were conducted in differentiating ES cells by using SirT1 overexpressing plasmid {pcDNA-3.1 SirT1, a kind gift from Dr. Hang Shi, Wake Forest University School of Medicine, North Carolina, USA (Yang et al., 2010)}. Data showed that SMC specific marker expressions were significantly increased upon overexpression of SirT1 during SMC differentiation (Figure 30A and B), suggesting that SirT1 over-expression can recapitulate the effects of miR-34a during SMC differentiation from ES cells. This finding was further confirmed by SirT1 agonist treatments. Resveratrol, a well-characterized SirT1 agonist (Borra et al., 2005), has been reported to promote osteogenic differentiation of mesenchymal stem cell (Shakibaei et al., 2012, Tseng et al., 2011) or phenotypic switching of VSMC (Thompson et al., 2014). The result from resveratrol experiments suggested that

resveratrol significantly increased SMC specific gene expression with the best concentration of 5 μ M (Figure 30C), further supporting SirT1 plays an important role during SMC differentiation.

To further confirm the hypothesis that SirT1 activation is required for miR-34a mediated SMC differentiation, control (pLL3.7-GFP) or miR-34a overexpressing (pLL3.7-GFP-miR-34a) ES cells were induced to differentiate into SMCs for 4 days then subjected to SirT1 specific inhibitor treatment. Data shown in Figure 31A revealed that inhibition of SirT1 alone significantly decreased SMC gene expression, while miR-34a overexpression up-regulated SMC marker. More importantly, inhibition of SirT1 almost completely abolished SMC specific gene up-regulation induced by miR-34a overexpression. Such finding was further confirmed by SirT1 knockdown experiments using SirT1 specific siRNA, suggesting that miR-34a up-regulates SMC gene expression through activation of SirT1 (Figure 31B). Taken together, SirT1 is involved in SMC differentiation and the activation of SirT1 is critical for miR-34a induced SMC gene up-regulation.

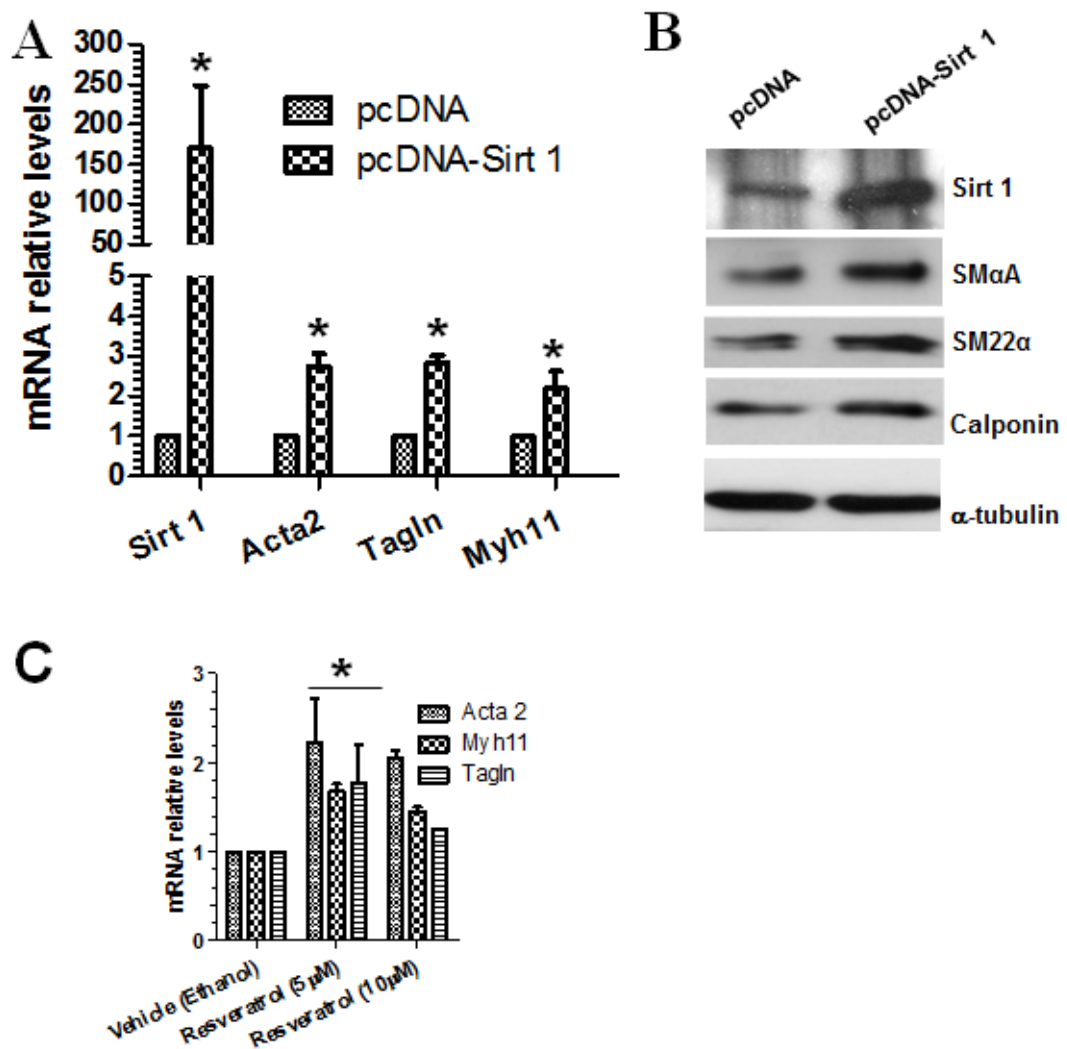


Figure 30. SirT1 overexpression or activation promotes SMC differentiation

(A and B) Day 2~3 differentiating ES cells were transfected with Sirt1 overexpressing (pcDNA 3.1-SirT1) or control (pcDNA 3.1) plasmids and cultured in differentiation medium for 48 to 72 hours. Cells were harvested and subjected to Real-Time PCR (A) or Western blot (B) analysis, respectively. (C) Resveratrol promotes SMC gene expressions. Undifferentiated ES cells were induced to SMC

differentiation for 3 days, then resveratrol or same amount of carrier vehicle (ethanol) was added into culture medium and incubated for further 24 hours. Cells were harvested and subjected to Real-Time PCR analysis. The data presented here are representative of mean \pm S.E.M. of three independent experiments. *P<0.05

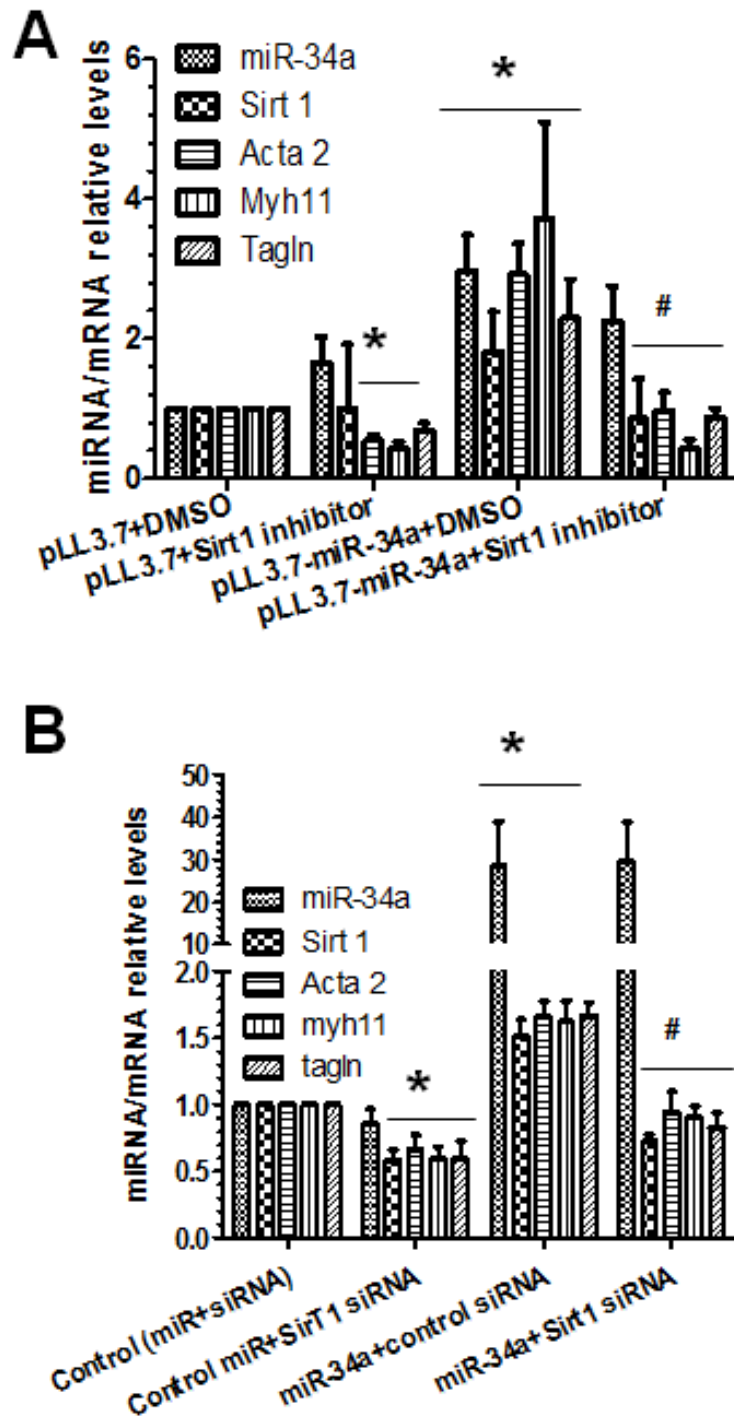


Figure 31 Inhibition/knockdown of SirT1 abolished SMC specific gene up-regulation induced by miR-34a overexpression

(A) Inhibition of SirT1 abolished SMC gene up-regulation induced by miR-34a

overexpression. Control (pLL3.7-GFP) or miR-34a overexpressing (pLL3.7-GFP-miR-34a) ES cells were induced to differentiate into SMCs for 4 days, then incubated with SirT1 specific inhibitor (500nM, EMD Millipore, 566322) or carrier vehicle (DMSO) for 6 or 12 hours. Total RNAs were harvested and subjected to Real-Time PCR analysis. (B) Knockdown of SirT1 eliminated the effect of miR-34a overexpression on SMC gene expression. Day 2 ~3 differentiating ES cells were co-transfected with miR-34a precursor, SirT1 siRNA or related control (including miRNA negative control and siRNA control), and cultured in differentiation medium for 48 hours. Cells were harvested and subjected to Real-Time PCR analysis. Data presented here are mean \pm S.E.M. of three to four independent experiments. *P<0.05 (versus related control); #P<0.05 (4th columns versus 3rd columns).

4.4.2 SirT1 regulates SMC transcription factors

SRF, MEF2C and Myocardin are well-known transcription factors for regulating SMC gene expression and cardiovascular system development. Results from this study have already suggested that miR-34a can regulate these transcription factors, and SirT1 activation is required for miR-34a mediated SMC gene up-regulation. To investigate whether SirT1 mediates SMC differentiation through regulation of these transcription factors and their underlying pathways, gene expression levels of SRF, MEF2c and Myocardin were examined in SirT1 overexpression experiment. Figure 32A revealed that by overexpressing SirT1, expression levels of SRF, MEF2c and Myocardin gene were significantly increased, suggesting SirT1 may have a direct role in regulation of these transcription factors during SMC differentiation. Such notion was further supported by SRF, MEF2c or Myocad gene reporter luciferase activity assays. Differentiating ES cells were transfected with SRF, MEF2c or Myocad gene reporter plasmids {pGL3-Luc-SRF, pGL3-Luc-MEF2c and pGL3-Luc-Myocd; Generated from previous study (Huang et al., 2013)} combined with SirT1 overexpression, and luciferase activity assays were conducted. The result showed that SirT1 overexpression significantly increased SRF, MEF2c and Myocd gene promoter activities (Figure 32B), suggesting that SirT1 may activate transcriptional activity of these three genes.

Furthermore, ChIP assays with SirT1 antibody were carried out to investigate whether SirT1 can directly bind to the promoters of SRF, MEF2c and Myocd, and the potential binding region(s) for SirT1 within these three gene promoters. A set of specific primers (4 pairs) spanning through the respective promoter regions of SRF, MEF2c and Myocad as described in previous study (Huang et al., 2013) were used in preliminary study and the best primer pairs were chosen in the following experiments. Figure 33 showed that SirT1 directly binds to the promoter regions between -1393 and -1274 of SRF gene, -1335 and -1263 of MEF2c gene and -708 and -620 of Myocd gene, respectively. It is also demonstrated that the binding activity was significantly enhanced by overexpression of SirT1. Taken together, these data strongly suggested that SirT1 transcriptionally regulates SMC transcriptional factor gene expressions through direct binding to the promoter regions of these genes during SMC differentiation from ES cells.

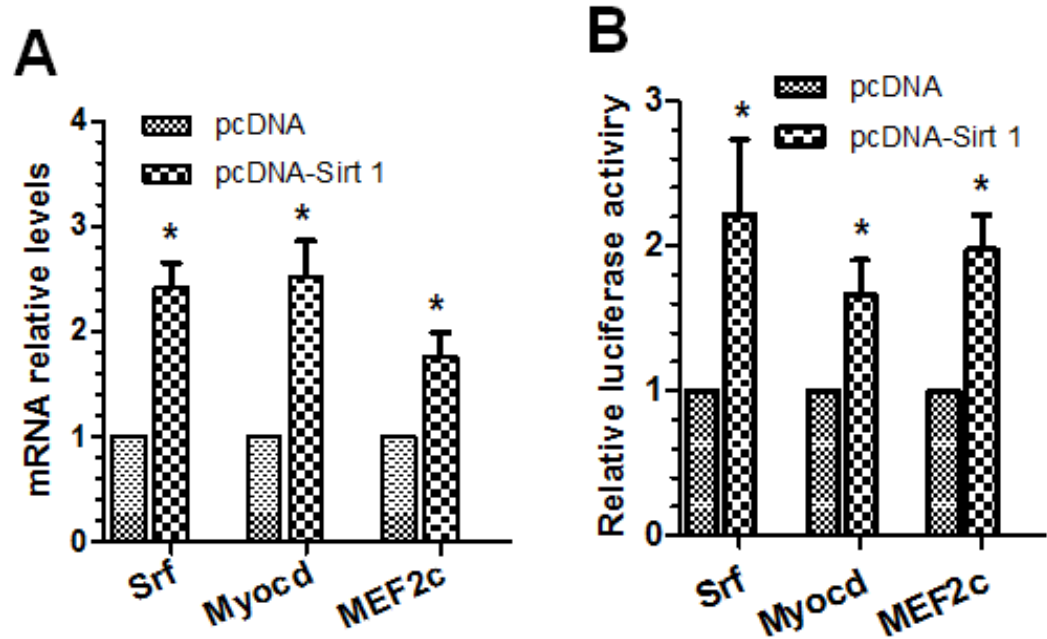


Figure 32 SirT1 up-regulates SMC transcriptional factors

Day 2 ~ 3 differentiating ES cells were transfected with control or SirT1 overexpressing plasmid (A), or combined with indicated luciferase reporter (B). Cells were harvested 48 hours after transfection and then subjected to Real-Time PCR (A) or Luciferase assay (B). (A) SirT1 overexpression up-regulates SRF, Myocd and MEF2c expression. (B) SirT1 overexpression regulates the promoter activities of SMC transcription factor genes. The data presented here are mean \pm S.E.M. of three to six independent experiments. * $P < 0.05$ (versus control)

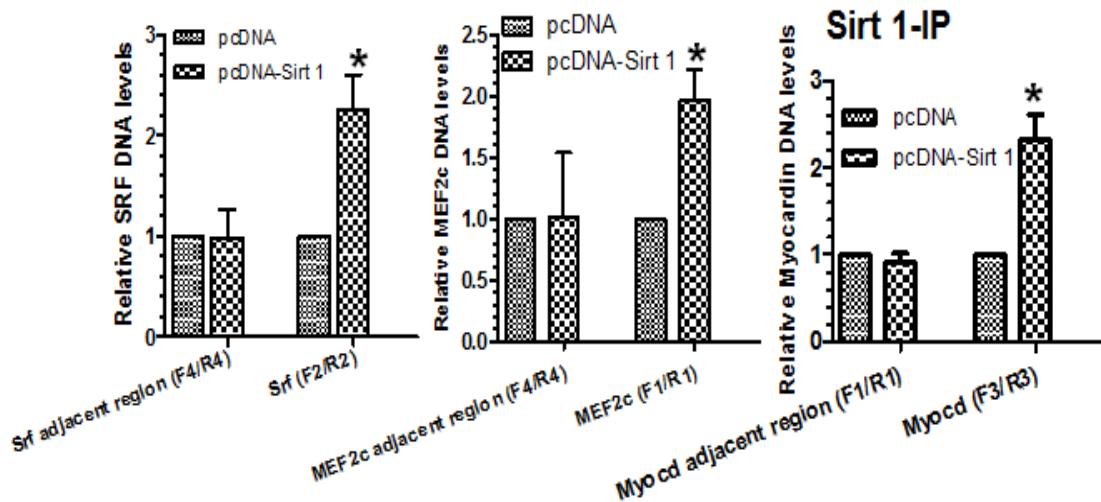


Figure 33 SirT1 directly binds to the promoter regions of SRF, MEF2c and Myocd genes.

Day 2 ~3 differentiating ES cells were transfected with control (pcDNA 3.1) or SirT1 overexpressing (pcDNA-3.1 SirT1) plasmids and cultured in differentiation medium for 48 hours. Cells were harvested then subjected to ChIP assays. ChIP assays were performed using antibody against SirT1 or normal rabbit IgG, respectively. Real-Time PCR amplifications of the adjacent regions were included as additional control for specific promoter enrichment. The data presented here are mean \pm S.E.M. of four independent experiments. *P<0.05 (versus control)

4.4.3 SRF binding site is required for SirT1 mediated SMC gene expression

SirT1 has been showed to up-regulate SMC specific gene expression and SMC specific transcriptional factors during SMC differentiation from ES cells. To investigate if SirT1 overexpression will activate specific SMC gene transcription, luciferase activity assay was performed with reporter plasmid of SM α A (pGL3-Luc-SM α A) and SM22 α (pGL3-Luc-SM22a) in differentiating ES cells, combined with SirT1 overexpression. Data from Figure 34 suggested that overexpression of SirT1 during SMC differentiation dramatically increased gene promoter activities of SM α A and SM22 α , indicating that SirT1 can activate specific SMC gene promoter. SRF is a well-known SMC transcriptional factors and SRF binding element (CArG) within promoter region of SMC genes is required for transcriptional activation of SMC gene expressions(Miano, 2003). As SirT1 has been showed to up-regulate SRF and SMC gene promoter gene expression during SMC differentiation, the functional involvements of SRF binding element within SMC gene promoter in SirT1 mediated SMC gene expression needed to be investigated.

To address this issue, luciferase assays using SRF binding site mutants (pGL3-Luc-SM α A-SRF^{mu} and pGL3-Luc- SM22a -SRF^{mu}) were carried out in differentiating ES cells combined with SirT1 overexpression. Data from Figure 34

showed that mutation of SRF binding element in pGL3-Luc-SM α A and pGL3-Luc-SM22 α caused complete loss of their transcriptional activity in response to SirT1 overexpression as compared with results from wild type reporter, suggesting SRF binding elements within SMC gene promoter region is responsible for SirT1 induced SMC gene up-regulation.

Moreover, ChIP assays were conducted using antibodies against SirT1 or SRF in differentiating ES cells to further verify if SirT1 activates specific SMC gene transcription through direct binding to their promoters. The result revealed that SirT1 directly bound to the region spanning around SRF binding element (CArG) of SM α A and SM22 α gene promoters, and such binding was dramatically reinforced by SirT1 overexpression (Figure 35A). Data from Figure 35B showed that SirT1 overexpression significantly enhanced the binding capacity of SRF to gene promoters of SM α A and SM22 α . Taken together, these finding demonstrated that SirT1 regulates specific SMC gene expression during SMC differentiation from ES cells through direct binding to the promoter region of SM α A and SM22 α , and such binding ability can also be further enhanced by SirT1 overexpression.

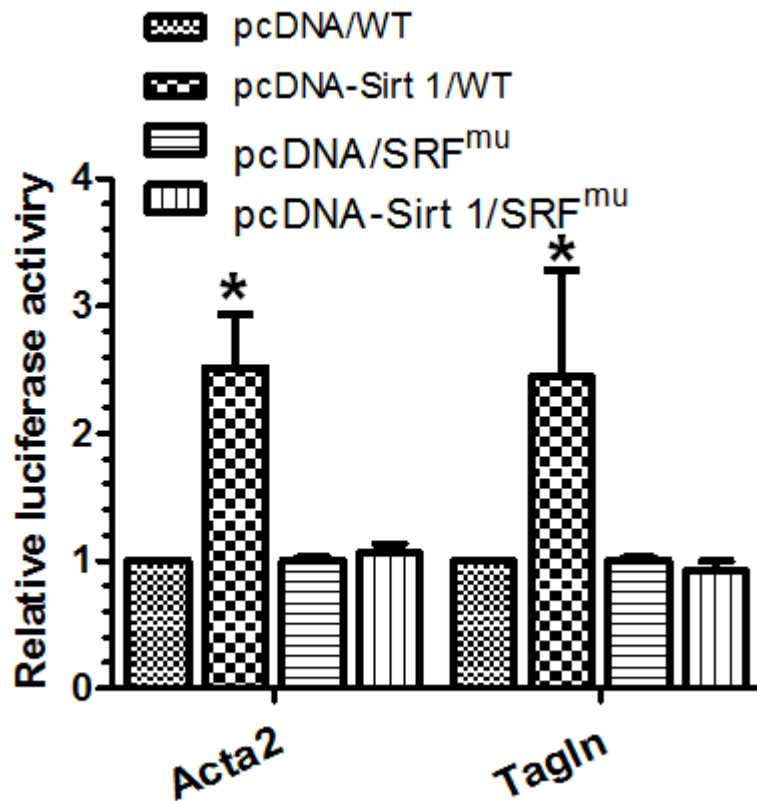


Figure 34 SRF binding site is required for SirT1 mediating SMC gene expression

Day 3~4 differentiating ES cells were co-transfected with wild type reporter (pGL3-Luc-SM α A and SM22 α pGL3-Luc-SM22a), or mutants (pGL3-Luc-SM α A- SRF^{mu} and pGL3-Luc- SM22a - SRF^{mu}), and SirT1 overexpressing or control plasmids as indicated. Luciferase assays were performed 48 hours after transfection. SirT1 overexpression significantly increases wild type reporter activities of SM α A and SM22 α gene, but not in the respective mutants. The data presented here are mean \pm S.E.M. of four independent experiments. *P<0.05 (versus control)

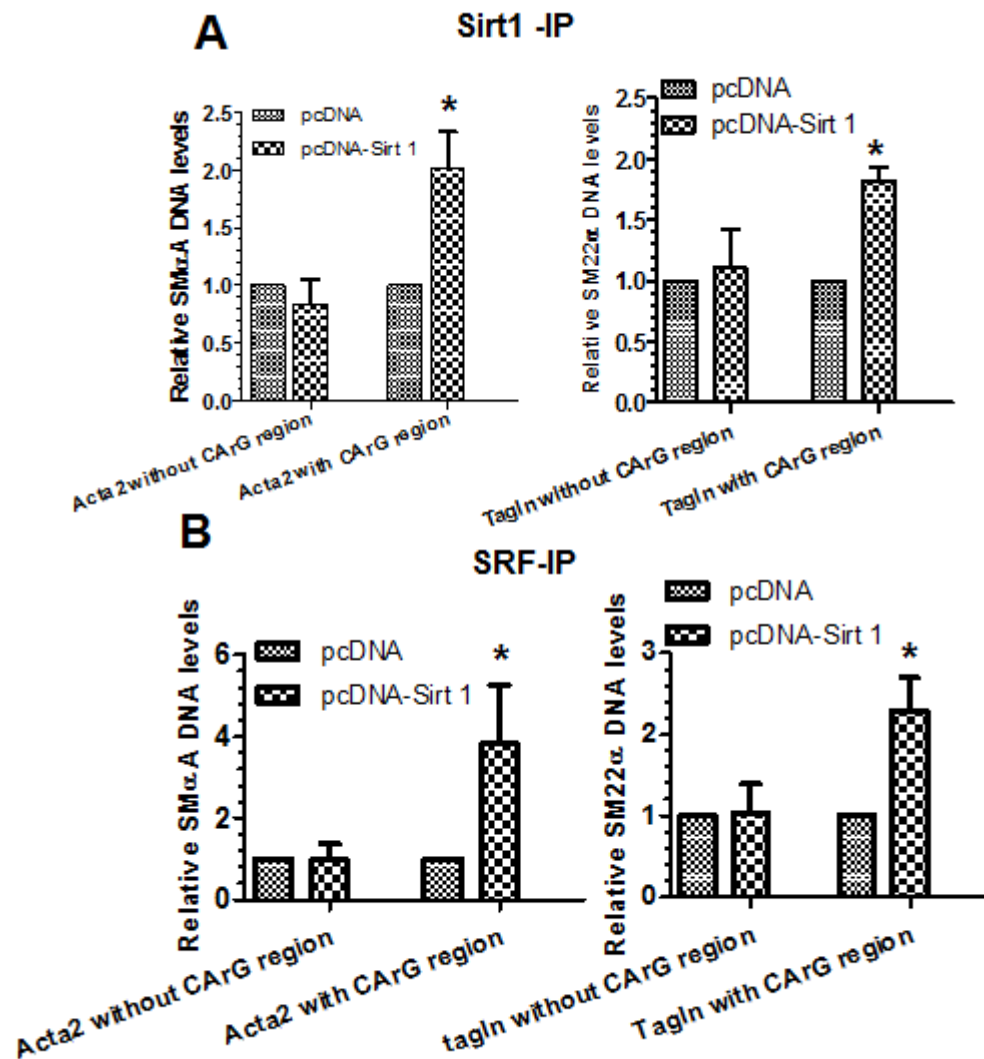


Figure 35 SirT1 directly binds to specific SMC gene promoter regions and increases the binding ability of SRF to SM α A and SM22 α

Day 2 ~3 differentiating ES cells were transfected with SirT1 overexpressing (pcDNA 3.1-SirT1) or control (pcDNA 3.1) plasmids and cultured in differentiation medium for 48 hours. Cells were harvested then subjected to ChIP assays using antibodies against SirT1(A) or SRF(B), as well as related IgG antibodies. (A) SirT1

directly binds to the regions spanning around SRF binding element (CArG) of SM α A and SM22 α gene promoters. (B) SirT-1 overexpression increases SRF binding to the promoter regions of SM α A and SM22 α genes. Real-Time PCR amplifications of the non-CArG regions were included as additional control for specific promoter DNA enrichment. The data presented here are mean \pm S.E.M. of four independent experiments. *P<0.05 (versus control)

4.4.4 SirT1 inhibits H3K9 methylation around SMC gene promoters

DNA and/or histone methylation is an important epigenetic gene regulation pathway. It is well-known that methylation of lysine H3K9 and H3K27 is closely associated with transcriptional repression (Rosenfeld et al., 2009), and SirT1 can regulate histone methylations in cancer (Liu et al., 2009, Liu and McCall, 2013). To investigate if SirT1 affects H3K9me3 expression levels during SMC differentiation, SirT1 overexpression experiments were conducted in differentiating ES cells and subjected to Western blot and Chip assays. Data showed that SirT1 overexpression had little effect on H3K9me3 protein expression (Figure 36A), however the enrichments of H3K9me3 on SM α A and SM22 α gene promoters with CArG regions were significantly inhibited by overexpression of SirT1 (Figure 36B), suggesting that SirT1 regulates SMC-specific gene expression at least partially through inhibiting H3K9 tri-methylation around SMC specific gene promoters.

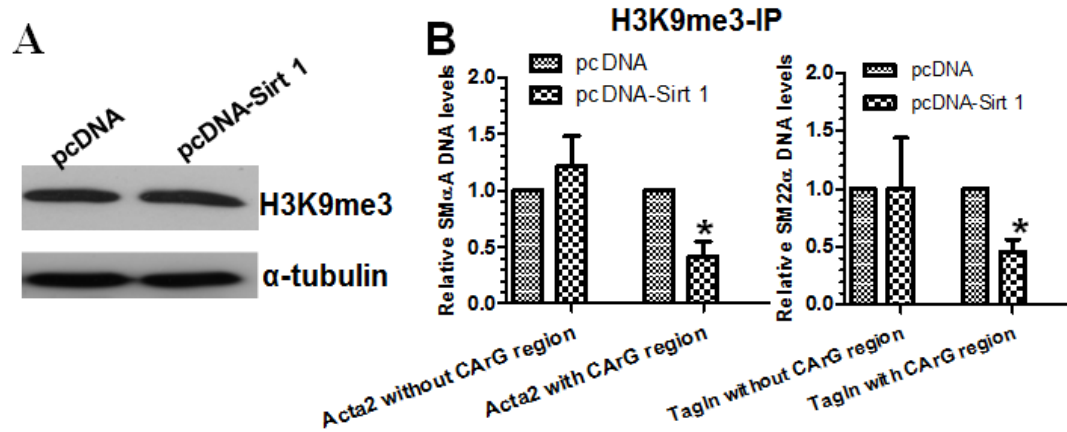


Figure 36 SirT1 promotes SMC gene expression through inhibiting H3K9me3 binding to SMC gene promoters.

Day 2~3 differentiating ES cells were transfected with control (pcDNA 3.1) or SirT1 overexpressing (pcDNA 3.1-SirT1) plasmids and cultured in differentiation medium for 48 hours. Cells were harvested and subjected to Western blot analyses (A) or ChIP assays (B), respectively. (A) H3K9me3 protein levels were not affected by SirT1 overexpression. (B) SirT1 overexpression reduces H3K9me3 binding to the promoter regions of SMαA and SM22α. ChIP assays using antibody against H3K9me3 or normal mouse IgG, respectively. Real-Time PCR amplifications of non-CArG regions were included as additional control. The data presented here are representatives or mean ± S.E.M. of four independent experiments. *P<0.05 (versus control)

CHAPTER 5 Discussion

The results obtained from this project advance our knowledge of the molecular mechanism mediating SMC differentiation by uncovering an important role of miR-34a in regulating SMC specific gene expression and SMC differentiation from mouse ES cells *in vitro* and *in vivo*. Such importance of miR-34a has also been confirmed in human SMC differentiation from stem cells. Surprisingly, SirT1 has been identified as the mRNA target of miR-34a, and the results demonstrated that miR-34a positively regulates its target gene, SirT1, during SMC differentiation from ES cells. Mechanistically, miR-34a is found to promote cell cycle arrest at G0/1 phase, and a significant decreased incorporation of miR-34a and SirT1 RNA into Ago 2-RISC complex was observed upon SMC differentiation. Also, SirT1 is defined as a potential transcription activator of SMC specific gene regulation. Furthermore, the SRF binding site(s) within SMC specific gene promoters is required for SirT1 mediated SMC gene transcriptional activation, and SirT1 regulates SMC transcription factors (SRF, MEF2c and Myocd) at transcriptional level. Finally, SirT1 has been observed to regulate SMC gene expression through modulating the histone methylation status around SMC specific gene promoters. Taken together, data provided in the current study strongly suggests that miR-34a is a powerful SMC differentiation modulator by positive regulating its target gene, *SirT1*, a molecule functioning as a potential SMC-specific gene transcriptional activator.

5.1 SMC differentiation from ES cells

ES cells are derived from the inner cell mass of a blastocyst stage embryo and hold the ability to differentiate various types of cells, including SMCs. SMCs play an important role in the embryonic development of cardiovascular system, as well as other organs and tissues. They also play critical roles in cardiovascular diseases, such as stroke, hypertension and atherosclerosis. Better understanding of the mechanism by which ES cells differentiate into SMCs not only contributes to extending our knowledge of cardiovascular system development and diseases, but also provides theoretical/technical information to the development of regenerative medicine and tissue engineering. SMC differentiation from stem cell is a complicated and poorly defined process regulated by various molecular signal pathways, which includes stimulation of ECM, growth factors, mechanical stress and other micro environmental milieu. Despite the process of SMCs differentiation from stem cells has been intensively studied, the understanding of mechanisms underlying SMC differentiation is still far from complete, especially in the fields of functional role of small non-coding RNAs, as revealed by recent studies.

In a previous study by Xiao et al. (2007) revealed that collagen type IV can stimulate ES cells to differentiate into sca-1 positive cells then further differentiate into SMCs, which is mediated by collagen IV-integrin $\alpha_1/\beta_1/\alpha_v$. In that study it was also

pointed out that collagen type I can potentially induce SMC differentiation from ES cells through similar pathway of collagen type IV. In the present study, one of the goals is to further confirming the hypothesis that similar to collagen type IV, collagen type I can also facility SMC differentiation from stem cells. Multiple SMC-specific markers including $\text{SM}\alpha\text{A}$, $\text{SM22}\alpha$, calponin and SM-MHC were applied in this project to examine if SMC differentiation has been realized. Determination of SMC differentiation from ES cells was carried out by detecting chosen SMC specific markers during differentiation. Data from Figure 6 showed that the gene expressions of SMC specific markers, including $\text{SM}\alpha\text{A}$, $\text{SM22}\alpha$, calponin and SM-MHC were significantly increased after 4 days into differentiation and reached maximum level at day 6, as compared to that of undifferentiated ES cells. mRNA levels of early SMC differentiation markers, such as $\text{SM}\alpha\text{A}$ and $\text{SM22}\alpha$ (Owens et al., 2004) were increased over 800 and 200 folds respectively, as compared with undifferentiated ES cells. Importantly, the expression levels of late/mature SMC differentiation markers including calponin and SM-MHC (Owens et al., 2004) over the differentiation period were over 40 times higher than that of undifferentiated ES cells. Nanog, OCT3/4 and KLF4 are key factors in maintaining self-renew, pluripotency and undifferentiated state of ES cells. As expected, the mRNA levels of these ES cell specific markers significantly decreased from day 2 of differentiation, and maintained over 50% reduction at day 8 of differentiation (Figure 7).

SRF, MEF2c and myocardin are well known SMC transcriptional factors and play critical roles in SMCs differentiation and/or mature SMCs phenotype switching. SRF can directly bind to CArG elements within the promoter regions of SMC specific gene, and recruits its cofactor: myocardin, to form SRF-myocardin complex and work together with other transcription factors to regulate SMC differentiation (Cao et al., 2005, Dressel et al., 2001, Pagiatakis et al., 2012, Wang et al., 2003, Margariti et al., 2009, Owens et al., 2004, Wang et al., 2004, Xiao et al., 2010). It has been reported that SRF, MEF2c and myocardin were activated during collagen IV induced SMC differentiation (Margariti et al., 2009, Huang et al., 2013). Real-Time PCR analysis (Figure 8) showed that these transcriptional factors can also be triggered by collagen type I during SMC differentiation from ES cell. mRNA level of SRF and MEF2c started to increase from day 4 and reached maximum at day 6, while that of myocardin increased at a slightly later time but also significantly up-regulated at day 6 and expressed maximally at day 8.

Protein levels of SM α A and calponin during SMC differentiation were detected by using Western blot analyzes, as presented in Figure 8. Both SM α A and calponin protein expressions were not detected at day 0 (undifferentiated ES cells), but strong signals were detected from day 6 samples. The protein expression of SM α A and

calponin appeared at slightly later time, as compared with mRNA level. These time differences in gene and protein expression are possible due to late translation from SM α A and calponin mRNA.

Taken together, these data suggested collagen type I may act as similar function of collagen type IV in SMC differentiation from ES cell, further supporting the findings from Xiao et al. (2007). Furthermore, Real-Time PCR result showed that undifferentiated stem cell markers were significantly down-regulated from day 2, which indicated that the early SMC differentiation from ES cell may start after 2 days into the SMC differentiation model. It is further confirmed by the results from gene expression level of SMC specific markers, which started to up-regulate from day 2, increased significantly at day 4 and reached maximum level at day 6. These findings suggested that day 2 to day 4 is the critical period in the SMC differentiation model. Based on this finding, most of the treatments in this project were carried out during this time period to investigate the mechanism by which miR-34a/SirT1 regulate SMC differentiation.

Furthermore, human ES cells were seeded on collagen coated flasks to examine whether collagen can stimulate SMC differentiation *in vitro* in a similar manner of that in mouse ES cells. Data from Figure 18A and B suggested that SMC specific

markers, including PDGFRB, were significantly up-regulated during upon collagen stimulation over 9 days. SM α A is one of the actins isoforms and major constituent of the contractile apparatus within SMCs. Immunofluorescence analysis from Figure 18C established the presence of SM α A in differentiated SMCs from human ES cells, which has been further confirmed by flow cytometry analyses (Figure 18D). These data clearly suggests that Collagen can initiate SMC differentiation from both mouse and human ES cells.

5.2 miR-34a and SMC differentiation

Accumulating evidences suggest that microRNAs play an important role in biological regulation. As described before, Dicer and Drosha are essential for biogenesis of mature miRNAs. Recent studies using Dicer or Drosha deficient ES cells which were unable to generate mature miRNAs demonstrated that microRNAs can regulate self-renewal and differentiation of ES cells (Murchison et al., 2005, Wang et al., 2007). Some miRNAs, i.e. miR-21, has been identified to directly target Nanog and Sox2 which are key factors for maintaining stem cell functions (Murchison et al., 2005, Houbaviy et al., 2003).

It has been reported that some miRNAs are involved in SMCs migration,

proliferation, phenotype switching and differentiation. Albinsson et al. (2010) reported that deletion of Dicer in vascular smooth muscle caused decreased SMC proliferation and differentiation, which suggested the important roles of miRNAs in VSMCs. Recently, miR-143 and miR-145 have been reported to play critical roles in SMC proliferation, phenotype switching and differentiation (Elia et al., 2009, Cordes et al., 2009, Cheng et al., 2009). Li et al. (2013a) reported that miR-638 inhibited SMC proliferation and migration through targeting Neuron-derived orphan receptor 1. However, the understanding of miRNAs in ES cells differentiation, especially in SMC differentiation is far from complete.

To identify the potential miRNAs involved in SMC differentiation from ES cells, microRNA microarrays analysis was carried out on undifferentiated ES cells, differentiating ES cells (4 days in differentiation conditions) and differentiated ES cells (8 days in differentiation conditions) in this project. Among the increased miRNAs, miR-34a was significantly up-regulated during SMC differentiation (Table 3), which was further confirmed by Real-Time PCR analysis (Figure 10), implying that miR-34a may also be involved in SMC differentiation.

In mammals, the miR-34 family comprises three processed miRNAs that are encoded by two different genes: miR-34a is encoded by its own transcript, and

miR-34b and miR-34c share a common primary transcript (Hermeking, 2010). In 2007, several laboratories independently reported that members of the miR-34 family are direct p53 targets, and their up-regulation induces apoptosis and cell-cycle arrest (He et al., 2007, Raver-Shapira et al., 2007, Chang et al., 2007). Strikingly, the most highly enriched gene ontology category among the upregulated transcripts induced by miR-34a overexpression was “cell cycle” (Chang et al., 2007), which strongly indicates that miR-34a is a major player in the regulation of cell cycle progression. Considering cell cycle arrest or exiting from cell cycle progression is a critical process during cell differentiation, miR-34a could be a major regulator in cell differentiation from stem cells. In addition, similar to other p53-target genes, miR-34a may be an important regulator for other signaling pathways involved in normal embryos development, cell differentiation, apoptosis, senescence, proliferation and various diseases including cancer and cardiovascular diseases. Importantly, it has been recently reported that suppression of somatic cell reprogramming into pluripotent cells by miR-34a was at least in part due to repression of pluripotency genes, including Nanog, Sox2 and N-Myc (Choi et al., 2011), which strongly suggests that miR-34a could play an important role in stem cell differentiation. Evidently, recent studies have suggested an important role of miR-34a in neural differentiation from neural stem cells (Aranha et al., 2011) and megakaryocytic differentiation from bipotent K562 human leukemia cells (Navarro

et al., 2009). miR-34a has also been identified as a critical cell-fate determinant in early-stage dividing colon cancer stem cells (Bu et al., 2013). However, the function role of miR-34a in SMC differentiation remains to be investigated.

To explore the functional role of miR-34a in SMC differentiation, miR-34a gain/lost-function analyses were carried out in differentiating ES cells *in vitro* by using Pre-miR™ mmu-miR-34a miRNA Precursor, Anti-miR™ miR-34a inhibitor and siPORT™NeoFX™ transfection agent. Data from Figure 11A showed that miR-34a expression was significantly up-regulated by miR-34a precursor, and miR-34a inhibitor knockdown the expression of miR-34a during SMC differentiation (Figure 12A). More importantly, gene expression of SM α A, SM22 α , calponin and SM-MHC were considerably increased upon miR-34a overexpression, while knockdown of miR-34a inhibited expression of these SMC specific markers Figure 11A and Figure 12A). Protein level of SM α A and SM-MHC was also up-regulated by miR-34a overexpression and down-regulated by miR-34a inhibition, respectively (Figure 11B and Figure 12B). It has been reported that miR-145 is a well-known SMC differentiation related miRNA (Cheng et al., 2009, Cordes et al., 2009, Elia et al., 2009). Effect of miR-145 precursor or inhibitor on SMC differentiation was also included in the respective experiments as positive control for the functional involvement of miRNAs in SMC differentiation. As described earlier, SRF,

myocardin and MEF2c are well-established SMC transcriptional factors. Data from Figure 13 and Figure 14 demonstrated that the expression levels of these SMC transcription factors were also positively regulated by miR-34a, suggesting these molecules work in concert during SMC differentiation.

Importantly, data shown in Figure 19 revealed that miR-34a over-expression significantly up-regulated, while miR-34a inhibition dramatically down-regulated SMC marker gene expressions in differentiating human ES cells, further demonstrating a critical role of miR-34a in mouse as well as human SMC differentiation from stem cells.

Furthermore, control (pLL3.7-GFP) or miR-34a overexpressing (pLL3.7-GFP-miR-34a) ES cell line were generated by using respective lentivirus in this study to investigate the potential roles of miR-34a in *in vivo* SMC differentiation. The *in vitro* experiments with these cell lines (Figure 15A and Figure 15B) showed that the miR-34a level in miR-34a overexpressing ES cell was slightly higher than that of control cells at undifferentiated stage, but significantly increased after 8 days into SMC differentiation. Meanwhile more SMαA positive cells were detected in miR-34a overexpression cells compared with that in control cells (Figure 15C). The function of miR-34a during SMC differentiation *in vivo* was examined by using

miR-34a overexpressing ES cell lines combined with *in vivo* Matrigel implantation model as described earlier. Data from Figure 16 showed that SM-MHC and GFP positive cells were detected in the Matrigel implants, and miR-34a overexpressing ES cells generate more SM-MHC positive cells compare with that from control ES cell line, which indicated that these exogenous ES cells, especially miR-34a overexpressing ES cell line, successfully differentiated towards SMCs *in vivo*. SMC differentiation of ES cells *in vivo* was further confirmed by Real-Time PCR and Western blot analyses, and results showed that miR-34a overexpression significantly up-regulated the expression of SMC specific markers (Figure 17).

Taken together, the results from this project provide clear evidence to support that miR-34a can regulate SMC markers expression in collagen induced SMC differentiation from both mouse and human ES cells *in vitro*. Also, by using miR-34a overexpressing cell lines and Matrigel implantation, miR-34a was shown to play an important role *in vivo*. These observations clearly implied that miR-34a is a SMC differentiation regulator.

5.3 Up-regulation of SirT1 by miR-34a during SMC differentiation is a specific event

By using computational algorithmic databases, SirT1 was predicted to be one of the top potential mRNA targets for miR-34a. Figure 21A showed two highly conserved binding sites for 5' end of miR-34a were detected on 3'UTR of SirT1. It has been suggested that miR-34a regulates cell cycle progress and apoptosis in cancer cells by repressing its target gene *SirT1* and forming a double positive feedback loop to regulate p53 activity (Yamakuchi et al., 2008, Yamakuchi and Lowenstein, 2009). Surprisingly, results from this project provided clear and solid evidence to suggest that rather than translational repression miR-34a regulates SirT1 in a translational activation manner, which likely represents a specific event between miR-34a and SirT1 during SMC differentiation from ES cell.

Such notion has been supported by several lines of evidence: First, SirT1 gene expression and miR-34a expression were significantly activated during SMC differentiation from ES cells (Figure 20A and Figure 10). Secondly, SirT1 gene and protein expression levels were positively regulated by miR-34a overexpression experiments, but down-regulated by inhibition of miR-34a during SMC differentiation (Figure 20B and C). Also, inhibition or knockdown of SirT1 in differentiating ES cells significantly abolished the up-regulation of SMC specific

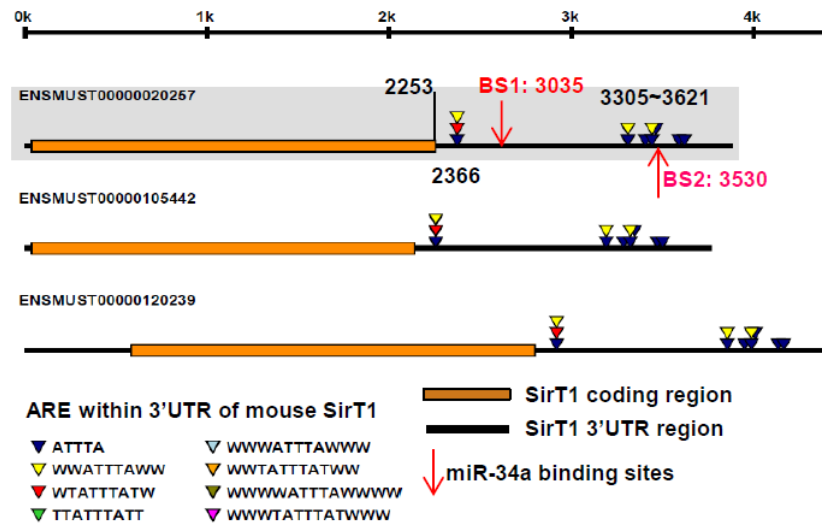
makers induced by miR-34a overexpression (Figure 31). Thirdly, the luciferase activity of wild type SirT1 3' UTR reporter was significantly up-regulated by miR-34a overexpression, but such up-regulation was completely abolished when miR-34a binding sites were mutated, which suggested that binding between miR-34a and SirT1 is required for miR-34a mediated SirT1 regulation (Figure 21). Fourthly, the luciferase activity of ZEB1 or MECP2 3'UTR reporters, which containing binding site(s) for non-miR-34a miRNAs (MECP2 is the mRNA target for miR-22, and ZEB1 is the mRNA target for miR-200C/150, respectively), cannot be regulated by miR-34a (Figure 24). Finally SirT1 gene expression levels in the Matrigel implants with miR-34a overexpressing ES cells were much higher than that of Matrigel plugs implanted with control ES cells, suggesting that SirT1 gene expression level is also positively associated with miR-34a expression level during SMC differentiation from ES cells *in vivo* (Figure 16 and Figure 17).

As described earlier, the classic thinking of miRNA regulation is that miRNAs bind to their target gene mRNAs and induce translational repression and/or degradation. The novel finding by which miR-34a positively regulates its target gene, SirT1, could be due to the following facts and/or observations:

1. It has been reported that cell cycle regulation is closely associated with

differentiation of stem cell (Pauklin and Vallier, 2013, White and Dalton, 2005). Figure 26 showed that differentiating ES cells undergo cell cycle arrest at G0/1 phase. As mentioned before, the miRNA-target gene regulatory machinery will switch from translation repression to activation when the cells have arrested at G0/1 phase and locked in quiescent cells (Vasudevan et al., 2007, Vasudevan et al., 2008). Therefore, the findings that miR-34a positively regulates target gene SirT1 during SMC differentiation is consistent with their data.

2. Another suggested criteria by Vasudevan et al. (2007) for miRNAs up-regulate their target genes is presence of AU-rich elements within 3'UTR of the target genes. By using free online search tool (<http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi>), similar to TNF- α we have found that SirT1 3'UTR is an A/U rich sequence (67%), and identified nearly 11 AU rich elements (ARE) sites within 3'UTR of SirT1 (spanning through 1000bps). More importantly, miR-34a binding site 2 is found to located closely with 8 of 11 ARE sites within SirT1 3'UTR (Figure 37).



<http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi>

Figure 37 The predicted ARE regions and miR-34 binding sites within mouse SirT1 3'UTR are depicted in this illustration

- While ES cells remain in undifferentiated stage, they have a shorter G1 phase as compared to differentiating ES cells. Interestingly, miR-34a negatively regulates SirT1 gene expression in undifferentiated ES cells, while positively regulates SirT1 gene expression in the terminally differentiated SMCs, as shown in Figure 23.
- Figure 28 showed that incubation of differentiating ES cells with 2µg/ml aphidicolin, a G0/G1 cell cycle arrest inducer, significant increased cell cycle arrest at G0/G1 phase, and such arrest was further enhanced by miR-34a overexpression. More importantly, aphidicolin could up-regulate SirT1 gene

reporter activity, and a synergetic effect between aphidicolin and miR-34a over-expression on SirT1 gene regulation was observed in this study.

5. Removal of surrounding RNA sequence and structure of SirT1 3'UTR almost abolished the up-regulative effects of miR-34a on SirT1 3'UTR reporter activity (Figure 23).
6. Significantly decreased incorporation of miR-34a and SirT1 RNA into Ago-RISC complex was observed upon SMC differentiation from RNA-IP experiment using antibody against Ago 2 (Figure 29).

All these observations have provided a strong indication that miR-34a may positively regulate SirT1 gene expression through their binding sites within 3'UTR, which has been nicely proven by the finding that miR-34a binding site 2, rather than binding site 1, is required for miR-34a mediated SirT1 gene up-regulation (Figure 21). Results from this project also clearly suggests that up-regulation of SirT1 by miR-34a during SMC differentiation is a specific event (G0/G1 cell cycle arrest) between miR-34a and SirT1 gene during SMC differentiation, and both miR-34a binding sites and surrounding RNA sequence/structures within 3'UTR of SirT1 gene are required for such an event.

Evidently, data from other studies also suggest that miRNAs can up-regulate their

target genes in other cell systems under normal cell culture condition. It has been reported that miR-155 directly and indirectly enhances its target gene *TNF- α* translation in macrophages (Tili et al., 2007), and miR-744 up-regulates CcnB1 through the binding sites within gene promoter region in NIH/3T3 fibroblasts as well as TRAMP C1 cells (Huang et al., 2011). Place et al. (2008) reported that miR-373 can target promoter sequences of E-cadherin and cold-shock domain-containing protein C2, and induces their gene expression. Additionally, miRNAs have also been suggested to up-regulate their target genes in immature oocytes (Mortensen et al., 2011).

5.4 SirT1 regulates SMC differentiation gene expression through a transcriptional mechanism

One of novel mechanistic findings in the project is that SirT1 regulates SMC differentiation gene expression through a transcriptional mechanism. SirT1 is a member of the a NAD⁺-dependent class III group of histone deacetylases, and has been reported to be involved in a variety of biological systems and cellular functions, including obesity-associated metabolic diseases, cancer, aging, cellular senescence, cardiac aging and stress, prion-mediated neurodegeneration, inflammation, and placental cell survival (Yang et al., 2013). Importantly, recent data also suggests that SirT1 is a critical mediator in regulation of various development genes during stem

cell differentiation (Calvanese et al., 2010), and plays an important role in various cellular differentiations including endothelial progenitor cells (Cheng et al., 2012), hematopoietic cells (Ou et al., 2011), and osteoblasts (Srivastava et al., 2012).

Results from this project provided clear evidence that SirT1 is an important SMC differentiation mediator by transcriptional regulation of SMC specific genes and transcriptional factors. Traditionally, SirT1 has mainly been linked to negative regulation of gene expression through deacetylation of histone and non-histone proteins (Tissenbaum and Guarente, 2001). However, accumulating evidence also strongly suggests that SirT1 can act both positively and negatively to control gene expression by recruiting a different set of coactivators and corepressors such as peroxisome proliferator-activated receptor γ coactivator-1 α (Rodgers et al., 2005) or via a transcriptional mechanism (Chang and Guarente, 2013). In response to stress, activated SirT1 transcriptionally regulates its downstream target genes such as transcriptional factors (forkhead box Os and hypoxia-inducible factors-2 α) and cardiac α -myosin heavy chain, through which SirT1 exerts a cardioprotection response (Yang et al., 2013). In consistent with these findings, overexpression of SirT1 by using SirT1 overexpressing plasmids or SirT1 agonist in differentiating ES cells positively regulates expression of SMC specific makers (Figure 30). More importantly, data from Figure 34 and Figure 32B clearly indicated that SirT1

up-regulated promoter activities of SMC specific gene or transcriptional factors (SM α A, SM22 α , SRF, MEF2c and myocd), which strongly demonstrated that SirT1 is a transcription activator for SMC differentiation genes. Direct evidence for such transcriptional regulation of SMC specific genes was obtained from ChIP assays (Figure 33 and Figure 35A), in which the data revealed that SirT1 can directly bind to the promoter DNA of SMC specific genes and transcription factors.

Another important finding of this project is that SirT1 acts as a potential transcriptional regulator for SMC gene regulation through modulating epigenetic modifications. It has been reported that SirT1 can induce the production of H3K9me3 in cultured U2OS cells (Vaquero et al., 2004) through directly interacting with deacetylating histone methyltransferase SUV39H1 (Vaquero et al., 2007). Data from Xiao et al. (2011) also suggested that H3K9 methylation was enriched within SMC specific gene promoters regions in the differentiating stem cells. H3K9me3 is well known for its repressive function in gene expression. Interestingly, data from Figure 36 revealed that instead of inducing H3K9me protein production, SirT1 represses H3K9 tri-methylation status within SMC-specific gene promoters, resulting in SMC gene activation. However, the functional involvements of SirT1 in the regulation of other epigenetic modifications remain to be fully elucidated.

CHAPTER 6 Conclusion, limitations and future plans

6.1 Conclusion

SMC differentiation from pluripotent stem cell plays critical roles in cardiovascular system development, diseases and tissue engineering. Despite the enormous efforts have been put into this field in the past decade, our understandings of the molecular mechanisms underlying SMC differentiation are still far from complete. miRNAs are small non-coding RNAs and their important functions in biological regulation had not been correctly recognized till recent years. The individual miRNAs and their functional roles in SMC differentiation from pluripotent stem cell remains to be further elucidated.

The present study have advanced our knowledge of the molecular mechanism mediating SMC differentiation by uncovering an important role of miR-34a in regulating SMC differentiation from mouse ES cells *in vitro* and *in vivo*, and provided compelling evidence to support the unexpected finding that miR-34a positively regulates its target gene SirT1 during SMC differentiation. This finding demonstrated that repression of SirT1 by miR-34a may switch to activation under specific conditions during the complex process of SMC differentiation, which

suggests that the “classical” miRNA-target gene repression regulatory machinery should be carefully considered under certain circumstance. Furthermore, the present study revealed that SirT1 regulates SMC gene expression through a transcriptional mechanism as well as an epigenetic signal pathway. Overall, the findings obtained from this study will significantly increase the understanding of the molecular mechanisms in SMC differentiation and benefit future application in regenerative medicine.

6.2 Limitation and further work

The limitation and further work are listed below:

1. Without any doubts, miR-34a plays an important role in SMC differentiation as supported by the compelling evidence presented in this study, however the functional importance of miR-34a in embryonic SMC differentiation and cardiovascular system development are still unclear. To fully address such points, global and/or VSMC-specific miR-34a knockout mice/embryos are required.
2. Results from this study revealed that miR-34a can also regulate SMC differentiation from human ES cells. However, the exact mechanism by which miR-34a regulates SMC differentiation from human ES cells needs to be further investigated. Also, data from the present study suggest that

miR-34a can up-regulate its target gene SirT1 during SMC differentiation from mouse ES cells, so experiments on human ES cells are required to investigate whether miR-34a can regulate SirT1 in a similar manner on human ES cells.

3. Although various mechanisms by which how miR-34a up-regulates its target gene SirT1 have been nicely proposed and demonstrated in this study, additional molecular mechanisms which are potential responsible for SirT1 up-regulation by miR-34a are possible existed and remain to be fully examined. For instance, whether RNA binding proteins are involved in up-regulation of SirT1 by miR-34a during SMC differentiation warranted for future investigation.
4. SirT1 has been nicely demonstrated for the first time to regulate SMC-specific genes and transcription factors through direct binding to the promoter regions within respective genes, however the minimal essential binding elements of SirT1 or exact SirT1 binding motifs within these gene promoter regions remains to be further identified
5. H3K9 trimethylation has been identified as one of mechanisms by which SirT1 regulates SMC differentiation gene expression, however, its functional involvements in the regulation of other epigenetic modifications remain to be fully elucidated

6. The down-stream signal pathways by which how miR-34a mediates SMC differentiation from stem cells have been nicely elucidated, however, the up-stream pathway(s) about how and why miR-34a is activated during SMC differentiation is still lacking.

Chapter 7 Appendix and reference

7.1 Appendix

7.1.1 Primer sets used in the project

Gene names	Forward (5'-3')	Reverse (5'-3')	Application
18s	CCCAGTAAGTGCGGGTCATAA	CCGAGGGCCTCACTAAACC	Real-time RT-PCR
U6 snoRNA	gatgacagcgaattcgtg	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR
miR-34a (mu/hu)	GGCAGTGTCTTAGCTGGTTGT	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR/RNA -IP
SMaA	TCCTGACGCTGAAGTATCCGAT	GGCCACACGAAGCTCGTTATAG	Real-time RT-PCR
SM22a	GAT ATG GCA GCA GTG CAG AG	AGT TGG CTG TCT GTG AAG TC	Real-time RT-PCR
h1-Calponin	GGT CCT GCC TAC GGC TTG TC	TCG CAA AGA ATG ATC CCG TC	Real-time RT-PCR
SM-myh11	AAG CAG CCA GCA TCA AGG AG	AGC TCT GCC ATG TCC TCC AC	Real-time RT-PCR
SMaA (hu)	TGAGCGTGGCTATTCCTTCGT	GCAGTGGCCATCTCATTTTCAA	Real-time RT-PCR
SM22a	GGCTGAAGAATGGCGTGATT	TCTGCTTGAAGACCATGGAGG	Real-time RT-PCR
h1-calponin (hu)	ATTTTGTAGGCCAACGACCTG	CCCACGTTACCTTGTTCCT	Real-time RT-PCR
SM-myh11 (hu)	GCGTCCATGCCAGATAACACA	TACCACATCTGCCCCAACCTT	Real-time RT-PCR
PDGFR-β (hu)	GCAATGTGACGGAGAGTGTGAA	AGCAAATTGTAGTGTGCCACC	Real-time RT-PCR
SRF	CCTACCAGGTGTCGGAATCTGA	TCTGGATTGTGGAGGTGGTACC	Real-time RT-PCR

Myocd	TCAATGAGAAGATCGCTCTCCG	GTCATCCTCAAAGGCGAATGC	Real-time RT-PCR
MEF2C	AAGCCAAATCTCCTCCCCCTAT	TGATTCAGTATGGCATCGTGT	Real-time RT-PCR
SirT1	GCTCGCCTTGCGGTGGACTT	GACGGCTGGAAGTGTCCGGG	Real-time RT-PCR/ RNA -IP
SM α -P1	CATAACGAGCTGAGCTGCCTC	CCAAACAAGGAGCAAAGACG	CHIP assay (with CArG region)
SM α -P2	GATCAGAGCAAGGGGCTATA	CTACTTACCCTGACAGCGAC	CHIP assay (without CArG region)
SM22 α -P1	GCAGGTTTCCTTTGTCGGGCCA	CTGCTTGGCTCACCACCCCG	CHIP assay (with CArG region)
SM22 α -P2	CTTTAAACCCCTCACCCAGC	ATGACTTGCACTTACAAGG	CHIP assay (without CArG region)
SRF-P_F2/R2	CCTGGCTGGCTTGGCACTCAC	ATCTGGCCGGACGGTGTGATA	CHIP assay
SRF-P_F4/R4	CCCTCTTCTGCCCTGCAGTCCT	CCGCGATTCCGTGGGAGGGA	CHIP assay (Adjacent region)
MEF2c-P_F1/R1	AGTCAGGACAGTGGCTTAGCGT	CGGGTTAGTCTCTGACAGTGGACC	CHIP assay
MEF2c-P_F4/R4	GCTCCAGTGTGCGATGTGCCA	TCCGCAGCAAGCCAGTTTCC	CHIP assay (Adjacent region)
Myocd-P_F1/R1	AGGCAGCCTGTTGTAGGCTCG	GAGAGGAGGTGGAGCCCTGCT	CHIP assay (Adjacent region)
Myocd-P_F3/R3	CGGGAGTTGCAAGCCAACCCA	TCCCCAGCTTACTGCAGGGCT	CHIP assay
pmiR-Luc-SirT1-W T	GGACTCACGCGTTGCTCCACCAGCAT TGGGAACT	CACCTGGAGCTCCTGCATTCTTTAG TGTAGCC	SirT1 3'UTR reporter clone
pmiR-Luc-SirT1-B S1 ^{mu}	CACCACAAAT GACTTTGAGA TGTGAATATG C	G CATATTACACA TCTCAAAGTC ATTTGTGGTG	miR-34a binding site 1 mutation
pmiR-Luc-SirT1-B S2 ^{mu}	CCCAGTTAGG ACCATTGACTTTGGAGGAGA AAAG	CTTT TCTCTCCAAAGTCAATGGT CCTAACTGGG	miR-34a binding site 2

			mutation
pmiR-Luc-SirT1-B S1/2 ^{mu}	CACCACAAAT GACTTTGAGA TGTGAATATG C	CCCAGTTAGG ACCATTGACTTTGGAGGAGA AAAG	miR-34a binding site 1 and 2 combinational mutation
SirT1 3'UTR miR-34a binding site 1	GAGCCTGCATAGATCTTCACCA	ACTCTCCCCAGTAGAAGTACCATT	RNA-IP
SirT1 3'UTR miR-34a binding site 1	TGCATAAAATACCCAGTTAGGACCA	GCACAAACACATCATGCAAATGG	RNA-IP
pmiR-Luc-miR-34a binding site	GCCTATGAGC ACCGTCA CTAGTGCGCA	TGCGCACTAG TGACGGT GCTCATAGGC	Generation of miR-34a artificial binding site

7.1.2 Microarray data from miR-34a overexpression

PROBE_ID	GENE SYMBOL	Fold changes	ACCESSION	PROTEIN_PRODUCT	Comment
Up-regulation by miR-34a over-expression					
ILMN_1221148	CNN1	5.622138271	NM_009922.3	NP_034052.2	h1-calponin
ILMN_2595612	Idh2	4.273697206	NM_173011.1	NP_766599.1	
ILMN_1244099	LOC100041388	4.014480491	XM_001476627.1	XP_001476677.1	
ILMN_2636803	Acyp2	4.003017615	NM_029344.3	NP_083620.1	
ILMN_1249378	Bhlhb2	3.909017089	NM_011498.4	NP_035628.1	
ILMN_1239370	Shfm1	3.745368624	NM_009169.2	NP_033195.1	
ILMN_2764781	Pls3	3.615617528	NM_145629.1	NP_663604.1	
ILMN_2971043	V1rd6	3.5419975	NM_030738.1	NP_109663.1	
ILMN_2695964	Lep	3.440290865	NM_008493.3	NP_032519.1	
ILMN_2641111	Dpagt1	3.275815373	NM_007875.2	NP_031901.2	
ILMN_2671366	6330577E15Rik	3.117077402	NM_026377.1		
ILMN_2599130	Aldoa	3.109562032	NM_007438.3	NP_031464.1	
ILMN_2777986	Pck1	3.074604817	NM_011044		
ILMN_1225414	Rab5c	2.914454097	NM_024456.2	NP_077776.2	
ILMN_2462745	H2-D1	2.798198849	NM_010380.3	NP_034510.3	
ILMN_2732848	TAGLN2	2.721118525	NM_178598.2	NP_848713.1	SM22alpha
ILMN_1245987	Hnrmpk	2.667151752	NM_025279.2	NP_079555.1	
ILMN_2654296	Rab10	2.617282273	NM_016676.4	NP_057885.1	
ILMN_2716838	Hspa4	2.350300909	NM_008300.3	NP_032326.3	
ILMN_2691613	Ugcg	2.334163401	NM_031868.2	NP_114074.1	
ILMN_2511089	Ppp1ca	2.300596168	NM_011673		
ILMN_2747959	Dcn	2.195035137	NM_007833.4	NP_031859.1	
ILMN_2607408	Pcbp4	2.178358316	NM_021567.2	NP_067542.2	
ILMN_2873319	Lypla1	2.173443856	NM_008866.2	NP_032892.1	
ILMN_2710354	ACTA2	2.1729868	NM_007392.2	NP_031418.1	SM α -actin
ILMN_2849521	Pycr1	2.169303834	NM_144795.2	NP_659044.1	
ILMN_1218842	LOC381105	2.160453784	XM_355021.1		
ILMN_2418957	5930418K15Rik	2.146499883			
ILMN_1254883	Fip1l1	2.143354544	NM_024183.4	NP_077145.2	
ILMN_2873822	AEBP1	2.079588497	NM_009636.1	NP_033766.1	aortic carboxypeptidase-like protein
ILMN_2643159	SIRT1	2.051813061	NM_019812.1	NP_062786.1	sirtuin 1
ILMN_2978040	Myh8	2.028195488	NM_177369.3	NP_796343.2	
ILMN_2876071	Fth1	2.020825185	NM_010239.1	NP_034369.1	
ILMN_2621581	SMTN	1.980577874	NM_013870.2	NP_038898.1	smoothelin
ILMN_1231072	A630082K20Rik	1.958463745	XM_145254		
ILMN_1218247	Palld	1.916824434	XM_974900.1	XP_979994.1	
ILMN_2988299	SRF	1.91038159	NM_020493.1	NP_065239.1	Serum response factor
ILMN_1227803	LOC674707	1.860780338	XR_031381.1		

ILMN_2836982	DES	1.821677099	NM_010043.1	NP_034173.1	desmin
ILMN_2435360	MYOCD	1.820363166	NM_145136.4	NP_660118.3	myocardium
ILMN_2691780	Myl3	1.786516369	NM_010859.1	NP_034989.1	
ILMN_2804261	CALD1	1.780141459	NM_145575.2	NP_663550.1	h-caldesmon
ILMN_2936221	Uppt	1.772344129	NM_001081189.1	NP_001074658.1	
ILMN_2758452	Hn1	1.75919755	NM_008258.1	NP_032284.1	
ILMN_1218347	MYLK	1.744983995	NM_139300.3	NP_647461.3	telokin
ILMN_2679063	Dctn2	1.735718025	NM_027151.1	NP_081427.1	
ILMN_2622217	MYH11	1.723761875	NM_013607.1	NP_038635.1	smooth muscle myosin heavy chain 11
ILMN_1214950	MEF2C	1.719307738	NM_025282.2	NP_079558.1	myocyte enhancer factor 2C
ILMN_2533447	AA407270	1.71203639	NM_001033208.3	NP_001028380.1	
ILMN_2518457	Irak1	1.708525698	NM_008363.2	NP_032389.2	
ILMN_2643326	2210020M01Rik	1.685217224	NM_183259.2	NP_899082.1	
ILMN_2699222	LOC669053	1.6652632	XM_972773.1	XP_977867.1	
ILMN_1230140	LOC385923	1.659273047	XM_359000.1		
ILMN_3133448	Mfge8	1.650499233	NM_001045489.1	NP_001038954.1	
ILMN_2694955	Igfbp4	1.611891845	NM_010517.2		
ILMN_1221235	4933425L21Rik	1.606305557	AK019858		
ILMN_1235646	1110069O07Rik	1.587316809			
ILMN_1234729	C130017115Rik	1.582996786	AK047868		
ILMN_2513536	Ythdf3	1.576358155	NM_172677.2	NP_766265.2	
ILMN_1227357	Flot2	1.573381621	NM_008028.2	NP_032054.1	
ILMN_1241039	2510042H12Rik	1.562091712	AK011092		
ILMN_1239535	Plekhn2	1.560872256	XM_924800.3	XP_929893.3	
ILMN_1212702	Hba-a1	1.547253336	NM_008218.2	NP_032244.2	
ILMN_1213431	LOC626583	1.530697304	XM_988307.1	XP_993401.1	
ILMN_2419138	VCL	1.501898958	NM_009502.4	NP_033528.3	metavinculin

Down-regulation by miR-34a over-expression					
ILMN_3005211	Akap6	0.628808485	NM_198111.2	NP_932779.2	
ILMN_1240938	AW212394	0.602759169	NM_133829		
ILMN_2684370	Igk-C	0.589605323	XM_132633.4		
ILMN_1213449	Man2c1	0.588938772	NM_028636.2	NP_082912.1	
ILMN_2653617	Cd63	0.588478696	NM_007653.1		
ILMN_1253322	Tex261	0.588088929	NM_009357.1	NP_033383.1	
ILMN_1244081	Rgs4	0.582896009	NM_009062.3	NP_033088.2	
ILMN_2744326	LOC665250	0.575666945	XR_033814.1		
ILMN_1253970	Sirt7	0.575421788	NM_153056.1	NP_694696.1	
ILMN_2791963	E2f6	0.563460644	NM_033270.1	NP_150373.1	
ILMN_2867147	Tyrobp	0.562853185	NM_011662.2	NP_035792.1	
ILMN_2714252	Kcnj3	0.557833702	NM_008426.1	NP_032452.1	
ILMN_1256518	Akr7a5	0.551937352	NM_025337.2	NP_079613.2	
ILMN_2752817	Myb	0.550583451	NM_010848.3	NP_034978.3	
ILMN_2607529	LOC100046044	0.546925564	XM_001475459.1	XP_001475509.1	
ILMN_1233283	P2rx5	0.543051384	NM_033321.1	NP_201578.1	
ILMN_1243319	Trib2	0.54076479	NM_144551.5	NP_653134.2	
ILMN_2626773	Rbak	0.533060729	NM_021326.2	NP_067301.1	
ILMN_1254199	Gaa	0.532515968	NM_008064.2	NP_032090.2	
ILMN_1235366	LOC100045628	0.53117846	XR_031850.1		
ILMN_2510612	Ywhaz	0.530641568	NM_011740.2	NP_035870.1	
ILMN_2628876	Jph2	0.528423701	NM_021566.1	NP_067541.1	
ILMN_1215067	Ctbp1	0.526071557	NM_013502.2	NP_038530.1	
ILMN_3090793	Nfyc	0.525698077	NM_008692.3	NP_032718.2	
ILMN_2752994	Fbxo32	0.52354856	NM_026346.1	NP_080622.1	
ILMN_2777474	H2-T23	0.519869076	NM_010398.1	NP_034528.1	
ILMN_2457437	A830080H07Rik	0.519543217			
ILMN_2537836	LOC385865	0.518471833	XM_358975.1		
ILMN_2927025	Sdcbp	0.517597012	NM_016807.1	NP_058087.1	
ILMN_1235635	Slco3a1	0.513386835	NM_023908.2	NP_076397.2	
ILMN_2705268	Atp5l	0.512445091	NM_013795.4	NP_038823.2	

ILMN_2529932	LOC385905	0.427319747	XR_034995.1	
ILMN_2710784	Gpsn2	0.426685111	NM_134118.3	NP_598879.1
ILMN_2618364	Flnc	0.426361591	NM_001081185.1	NP_001074654.1
ILMN_2715289	Exosc7	0.425933252	NM_001081188.1	NP_001074657.1
ILMN_2693976	Ncl	0.424817135	NM_010880.3	NP_035010.3
ILMN_1236828	Usp20	0.422404031	NM_028846.3	NP_083122.1
ILMN_1256369	Lynx1	0.419799355	NM_011838.4	NP_035968.1
ILMN_1249514	Large	0.415024023	NM_010687.1	NP_034817.1
ILMN_1226970	Wwp2	0.40899385	NM_025830.3	NP_080106.1
ILMN_2697918	LOC381302	0.408386585	XM_355244.1	
ILMN_1255927	Ubtf	0.404891052	NM_001044383.1	NP_001037848.1
ILMN_1221168	2310061F22Rik	0.404598175	NM_153775.1	NP_722470.1
ILMN_2681030	EG629595	0.402452137	XM_894495.2	XP_899588.1
ILMN_1237183	Ociad1	0.381976204	NM_023429.3	NP_075918.1
ILMN_2713060	Fam131a	0.369421669	NM_133778.2	NP_598539.1
ILMN_2733356	Endod1	0.364638976	NM_028013.2	NP_082289.1
ILMN_1252204	Sepw1	0.364105606	NM_009156.2	NP_033182.1
ILMN_1250816	Apeh	0.363210275	NM_146226.1	NP_666338.1
ILMN_1244098	Dst	0.359134991	NM_134448.2	NP_604443.2
ILMN_2445548	C330006A16Rik	0.358698462		
ILMN_1231542	Ttn	0.357513978	NM_011652.3	NP_035782.3
ILMN_1377923	Actb	0.353220505	NM_007393.3	NP_031419.1
ILMN_1254358	Igfbp5	0.348021168	NM_010518.2	NP_034648.2
ILMN_2527650	LOC268700	0.346186618	XM_193742.2	
ILMN_3073318	Tom1l2	0.346158034	NM_153080.2	NP_694720.2
ILMN_2654074	Sesn1	0.345735691	NM_001013370.1	NP_001013388.1
ILMN_2994779	Gpsn2	0.340700999	NM_134118.1	NP_598879.1
ILMN_2480021	0610007P22Rik	0.339814991	NM_026676.2	NP_080952.1
ILMN_1232298	BC003940	0.335811448	NM_001033231.2	NP_001028403.1
ILMN_1377918	LOC100048105	0.335515669	XM_001479832.1	XP_001479882.1
ILMN_2611699	Jmjd1b	0.335403769	NM_001081256.1	NP_001074725.1
ILMN_2661005	1110018G07Rik	0.326283053	NM_178065.3	NP_835166.2
ILMN_2723881	Hcfc1	0.326241686	NM_008224.3	NP_032250.2
ILMN_2737658	Pdk2	0.326102669	NM_133667.1	NP_598428.1
ILMN_1219978	Appl2	0.325859192	NM_145220.2	NP_660255.1
ILMN_3128792	Ttn	0.316510794	NM_028004.2	NP_082280.2
ILMN_2704562	LOC100047628	0.316406947	XM_001476703.1	XP_001476753.1
ILMN_1244316	Hbb-b1	0.31627096	AK010993	
ILMN_2621471	1110007L15Rik	0.31623635	NM_026269.1	NP_080545.2
ILMN_1214024	2310003M01Rik	0.315148721	AK009124	

ILMN_1232295	Sort1	0.509807516	NM_019972	
ILMN_1227459	Gga2	0.508942236	NM_028758.2	NP_083034.1
ILMN_2425415	Ube3b	0.505632053	NM_054093.2	NP_473434.2
ILMN_1239729	Kif1b	0.504351203	NM_207682.2	NP_997565.2
ILMN_2620078	Dap3	0.501323496	NM_022994.2	NP_075370.1
ILMN_2589073	Lrrc10	0.500021345	NM_146242.2	NP_666354.1
ILMN_1227839	Brwd1	0.499482347	NM_145125.2	NP_660107.1
ILMN_2599110	Uspl1	0.498577121	NM_001013378.1	NP_001013396.1
ILMN_1238117	LOC386054	0.493252261	XM_359045.1	
ILMN_2986315	Mir16	0.487658501	NM_019580.3	NP_062526.1
ILMN_1254987	Mbnl2	0.487128377	NM_175341.4	NP_780550.1
ILMN_2750428	Timm44	0.476772184	NM_011592.1	NP_035722.1
ILMN_1234324	Pdzd8	0.474702905	NM_001033222.2	NP_001028394.1
ILMN_2667447	Hdlbp	0.473999228	NM_133808.4	NP_598569.1
ILMN_1259528	LOC382092	0.469147057	XM_356174.1	
ILMN_2739843	Metrn	0.467418561	NM_133719	
ILMN_2993267	Keap1	0.465161445	NM_016679.2	NP_057888.1
ILMN_2971559	Eef1a2	0.465024364	NM_007906.2	NP_031932.1
ILMN_1257759	Tnrc6b	0.464889956	NM_144812.2	NP_659061.2
ILMN_2733046	Cdc25c	0.455919501	NM_009860.2	NP_033990.2
ILMN_1241915	Notch1	0.454971716	NM_008714.2	NP_032740.2
ILMN_2609948	Suds3	0.453359029	NM_178622.3	NP_848737.2
ILMN_1238313	Cnot2	0.452552762	NM_001037846.2	NP_001032935.1
ILMN_1247990	Purb	0.451824808	NM_011221.2	NP_035351.1
ILMN_1258682	Map2k2	0.451660818	NM_023138.3	NP_075627.2
ILMN_1220309	Cep63	0.451484232	NM_001081122.1	NP_001074591.1
ILMN_1247312	Kpna3	0.449438204	NM_008466.3	NP_032492.1
ILMN_2628875	Jph2	0.447043915	NM_021566.1	NP_067541.1
ILMN_1227926	Flt1	0.445619416	NM_010228.3	NP_034358.2
ILMN_1220498	Pygm	0.44414986	NM_011224.1	NP_035354.1
ILMN_2655260	Ptp4a3	0.442203662	NM_008975.2	NP_033001.2
ILMN_2930819	Acd	0.437936397	NM_001012638.1	NP_001012656.1
ILMN_2675785	Myo18b	0.433358207	XM_912851.3	XP_917944.3
ILMN_1221702	Plaa	0.432391343	NM_172695.2	NP_766283.2
ILMN_1246601	Glg1	0.431889168	NM_009149	
ILMN_2724545	Sbk	0.431232234	NM_145587.1	
ILMN_2813594	Zfp691	0.431018251	NM_183140.1	NP_898963.1
ILMN_2632665	Cav1	0.430594443	NM_007616.3	NP_031642.1
ILMN_2546596	2310076E21Rik	0.429992929	AK010195	
ILMN_2656677	Fkbp8	0.429677397	NM_010223.1	NP_034353.1
ILMN_2739760	Prelp	0.428983401	NM_054077.3	NP_473418.2
ILMN_2499264	4933428A15Rik	0.42785664	NM_027756	

ILMN_1224045	Dsp	0.31458421	XM_621314.3	XP_621314.2
ILMN_2699028	Crybg3	0.314261164	NM_174848.2	NP_777273.1
ILMN_2740852	F2r	0.306318637	NM_010169.3	NP_034299.2
ILMN_2670713	Cyfp2	0.305397666	NM_133769.2	NP_598530.2
ILMN_2777798	EG667723	0.294813005	XM_001001948.1	XP_001001948.1
ILMN_2590961	Hadh	0.294489032	NM_008212.3	NP_032238.2
ILMN_1213609	Txnip	0.294044329	NM_023719.1	NP_076208.1
ILMN_1246808	Serpine2	0.285704085	AK045954	
ILMN_2760295	BC003965	0.284864172	XM_128528.2	
ILMN_2762056	Dtnbp1	0.264900509	NM_025772.3	
ILMN_2710139	Ppargc1a	0.262055987	NM_008904.1	NP_032930.1
ILMN_2625981	Tcp11l2	0.252211623	NM_146008.2	NP_666120.1
ILMN_2591731	Ptprb	0.243202853	NM_029928.1	NP_084204.1
ILMN_2636335	Sfrs2	0.239533525	NM_011358.1	NP_035488.1
ILMN_2565729	B230386D16Rik	0.238450669	AK046451	
ILMN_2607880	Tkt	0.236974973	NM_009388.2	NP_033414.1
ILMN_2771991	Ndrp2	0.236307041	NM_013864	
ILMN_2538355	LOC386233	0.226558057	XM_359128.1	
ILMN_2588056	LOC100042427	0.22620383	XM_001478518.1	XP_001478568.1
ILMN_2593694	Lrtm1	0.225907269	NM_176920.3	NP_795894.1
ILMN_2635082	LOC620009	0.223555458	XR_030609.1	
ILMN_2747168	Ddx5	0.214385503	NM_007840.3	NP_031866.2
ILMN_2762863	AI606181	0.213847123	XR_035118.1	
ILMN_2788673	Pgm2	0.213514628	NM_028132.2	NP_082408.2
ILMN_2806676	Acads	0.205321587	NM_007383.2	NP_031409.2
ILMN_2918987	Lias	0.203012732	NM_024471.2	NP_077791.1
ILMN_1249486	Mgl1	0.194652521	NM_010796.2	NP_034926.1
ILMN_3144820	LOC329575	0.194506015	NM_001024849.1	NP_001020020.1
ILMN_2712957	Prpf6	0.194453201	NM_133701.2	NP_598462.1
ILMN_2673024	Mrps24	0.192982414	NM_026080.2	NP_080356.1
ILMN_1219840	2310033F14Rik	0.18445462		
ILMN_1237061	Dm15	0.183702219	NM_032418	
ILMN_2764727	Actn2	0.174128023	NM_033268.2	
ILMN_1236904	Mrpl23	0.164063753	NM_011288.1	NP_035418.1
ILMN_1215942	LOC381095	0.152240283	XM_358506.1	
ILMN_1239040	mtDNA_CytB	0.112099942		

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